AD-A101 009 LETTERMAN ARMY INST OF RESEARCH SAN FRANCISCO CA F/G 6/18
ASSESSMENT OF MUTAGENIC ACTIVITY IN THERMALLY PROCESSED, FROZEN--ETC(U)
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AD A101069

INSTITUTE REPORT NO. 70

ASSESSMENT OF MUTAGENIC ACTIVITY IN THERMALLY PROCESSED, FROZEN, ELECTRON-IRRADIATED, AND GAMMA-IRRADIATED BEEF USING THE AMES SALMONELLA/MAMMALIAN MICROSOME MUTAGENICITY ASSAY

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DIVISION OF RESEARCH SUPPORT

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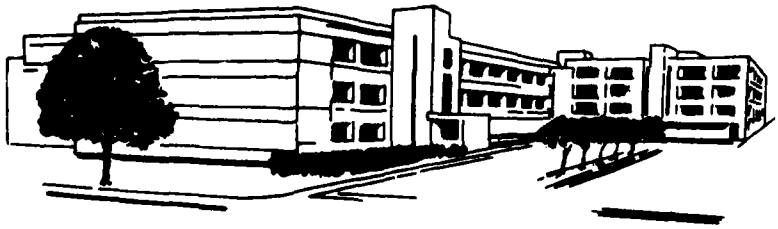

LETTERMAN ARMY INSTITUTE OF RESEARCH PRESIDIO OF SAN FRANCISCO CALIFORNIA 94129

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Assessment of Mutagenic Activity in Thermally Processed, Frozen, Electron-Irradiated, and Gamma-Irradiated Beef Using the Ames Salmonella/Mammalian Microsome Mutagenicity Assay--Guthertz and Fruin

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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: LAIR Institutute Report No.70
2. GOVT ACCESSION NO.: AD-A101009
3. RECIPIENT'S CATALOG NUMBER:

4. TITLE (and Subtitle): Assessment of Mutagenic Activity in Thermally Processed, Frozen, Electron-Irradiated, and Gamma-Irradiated Beef Using the Ames Salmonella/ Mammalian Microsome Mutagenicity Assay
5. TYPE OF REPORT & PERIOD COVERED: June 1978 - April 1980 Final
6. PERFORMING ORG. REPORT NUMBER:

7. AUTHOR(s): Linda S. Guthertz, MA
John T. Fruin, DVM, PhD, LTC, VC
8. CONTRACT OR GRANT NUMBER(s):

9. PERFORMING ORGANIZATION NAME AND ADDRESS: Division of Research Support
Letterman Army Institute of Research,
Presidio of San Francisco, CA 94129
10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: Project 3M762772A811 Military Nutrition & Food Hygiene
WU 039-Irradiated Foods Study

11. CONTROLLING OFFICE NAME AND ADDRESS: U.S. Army Medical Research and Development Command
Fort Detrick
Frederick, MD 21701
12. REPORT DATE: June 1981
13. NUMBER OF PAGES: 117

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office):
15. SECURITY CLASS. (of this report): UNCLASSIFIED
15a. DECLASSIFICATION/DOWNGRADING SCHEDULE:

16. DISTRIBUTION STATEMENT (of this Report)

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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Irradiated Foods, Ames Assay, Food Toxicology, LAIR

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Studies were undertaken to determine the mutagenic activity of beef that had been thermally processed, frozen, electron-irradiated, and gamma-irradiated. The Ames Salmonella/mammalian microsome mutagenicity assay, with several modifications, was used. Considerable difficulties in performing the test and interpreting the results were encountered. Experiments conducted showed that on some occasions up to 80% of the apparent revertants were not true revertants. The meats contained water-soluble growth factors, particularly

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histidine, which apparently supported greater than normal growth and macrocolony formation. Subsequently, the level of histidine in the media was reduced by an amount equal to that contributed by the meats. Also, extracts of the meat were substituted for whole meats as test material for evaluation. Particulate matter from the whole meats made automated colony counting impossible and complicated manual counting. Data collected failed to demonstrate that any of the meats or processing techniques produced mutagens. It was concluded that the test had limited applicability to whole food items and that the use of thermally, frozen, electron-irradiated, and gamma-irradiated processing does not induce mutagenic potential in beef.

ABSTRACT

Studies were undertaken to determine the mutagenic activity of beef that had been thermally processed, frozen, electron-irradiated, and gamma-irradiated. The Ames Salmonella/mammalian microsome mutagenicity assay, with several modifications, was used. Considerable difficulties in performing the test and interpreting the results were encountered. Experiments conducted showed that on some occasions up to 80% of the apparent revertants were not true revertants. The meats contained water-soluble growth factors, particularly histidine, which apparently supported greater than normal growth and macrocolony formation. Subsequently, the level of histidine in the media was reduced by an amount equal to that contributed by the meats. Also, extracts of the meat were substituted for whole meats as test material for evaluation. Particulate matter from the whole meats made automated colony counting impossible and complicated manual counting. Data collected failed to demonstrate that any of the meats or processing techniques produced mutagens. It was concluded that the test had limited applicability to whole food items and that the use of thermally, frozen, electron-irradiated, and gamma-irradiated processing does not induce mutagenic potential in beef.

PREFACE

The authors wish to thank LTC D. Hilmas, MAJ F. Chapple, Mr. C.D. Kuzdas, and Dr. H. Sauberlich for their assistance in experimental design and for reviewing the report. We wish to thank Dr. S. Taylor, Ms. E. Lieber, Mr. J. Dacey, Mr. W. Wise, Mr. P. Taylor, and SSG F. Pulliam for their assistance in performing the assays.

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DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:
SGRD-ULZ-QA

30 December 1980

MEMORANDUM FOR RECORD

SUBJECT: Report of GLP Compliance

This will certify that I have reviewed the written, dated and signed inspection records of Mr. William Wise, who performed quality assurance functions related to LAIR GLP study 78001 and have determined that inspections were made as follows:

14 Feb 79
23 May 79
25 May 79
31 May 79
19 Jun 79
28 Nov 79
30 Nov 79

Findings were reported to management 15 November 1979.

JOHN L. SZUREK
MAJ, MS
Quality Assurance Officer

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Signatures of Principal Scientists
Involved in the Study

We, the undersigned, believe the study described in this report to be scientifically sound and the results and interpretations to be valid. The study was conducted to comply, to the best of our ability, with the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies outlined by the Food and Drug Administration.

Linda S. Guthertz, M.A.
Principal Investigator

John T. Fruin, D.V.M., Ph.D.
LTC, VC
Study Director

INTRODUCTION

The testing of thermally processed, frozen, electron-irradiated and gamma-irradiated beef using the Ames assay was specified in the protocol entitled, "Animal Feed Protocol for Irradiation Sterilized Test Feeds", prepared by the Office for the Wholesomeness of Irradiated Foods, U.S. Army Medical Research and Development Command, dated 21 October 1975 (1). Modifications to the standard Ames assay were directed by staff members of the Food and Drug Administration at a meeting on 11 July 1978 in Washington D.C.

Test meats were supplied by the U.S. Army Natick Research and Development Command, Natick. They were from lots prepared and processed, September 1977 in accordance with the basic protocol(1). Processing procedures are summarized below. Beef carcasses were deboned and defatted using normal commercial procedures and cut into chunks weighing between 0.125 lb and 1.5 lb. For each 100 lb of meat 0.75 lb of NaCl, 0.375 lb of $NaPO_4$, and 3 lb chopped ice was added; and then mixing occurred. After mixing, the beef was stuffed into easy-peel casings. The beef was enzyme inactivated at 68-74C. The beef was canned for all processes except for the electron-irradiated, in which case the meat was flexibly packaged. Thermal processing was done at a sterilization value of not less than 5.8 at the slowest heating spot in the can. The frozen beef was maintained at -18 to -40C from time of production. The gamma-irradiated beef was irradiated in the U.S. Army Natick Research and Development Command Cobalt-60 facility. The dose range was 4.7 to 7.1 million rads (Mrad). The electron-irradiated beef was irradiated in the U.S. Army Natick Research and Development Command linear accelerator. The electrons were delivered in pulses of approximately 180 pulses per second. The electron energy spectrum was allowed to peak between 9 and 10 million electron volts (Mev) during irradiation of the beef with a full width of half maximum of 0.5 Mev or less.

Within the last few years, the use of microbial systems to screen substances for mutagenic potential has received considerable attention. Test procedures using the yeast, Saccharomyces cerevisiae, the fungus Neurospora, and the bacteria Salmonella typhimurium and Escherichia coli have been developed. Of these procedures, the Salmonella/mammalian microsome mutagenicity assay, developed by Ames and co-workers(2), has been used most extensively. This assay has demonstrated an approximate 90% accuracy in the prediction of a variety of carcinogenic chemicals as mutagens. It is equally accurate in predicting that a non-mutagenic compound is non-carcinogenic. Mutagens identified by this assay range from flame retardants used in fabrics to cigarette smoke condensate.

The short term mutagenesis assay developed by Ames et al (2)employs five mutant strains of Salmonella typhimurium designated TA98, TA100, TA1535, TA1537, and TA1538. Due to a specific mutation in the histidine operon, these strains are unable to grow in the absence of histidine. When grown on media containing a level of histidine sufficient for a few cell divisions, only cells able to revert to histidine independence are able to form colonies easily visible against the background lawn. Each of these mutant strains has a fairly constant rate of spontaneous reversion; however, the mutation frequency is significantly increased when a chemical mutagen is added to the system.

In addition to the previously described mutation within the histidine operon, the five tester strains contain additional mutations to increase their usefulness in the assay. All strains contain the rfa mutation which results in increased permeability of the cell wall allowing the entrance of more substances. All of the tester strains contain the uvrB mutation which results in defective excision repair of damaged DNA molecules. This mutation enhances the sensitivity of the strains to some mutagens. By incorporating all five tester strains into each assay, the type of mutation can be distinguished. Strain TA1535 is for detection of mutagens causing base-pair substitutions. Strains TA1537 and TA1538 are for detection of frameshift mutagens. Strains TA98 and TA100 were derived by the addition of a plasmid to strains TA1538 and TA1535, respectively. Carriage of this plasmid makes these two strains more sensitive to certain mutagens in addition to providing resistance to ampicillin.

Some mutagens are inactive unless they are metabolized to active forms. Induced liver enzymes possess the capability of metabolizing many of these compounds to their mutagenic form. For this reason, a mammalian microsomal activation system prepared from rat liver is included in the test.

Of the three formats for performance of the assay, the one most frequently used is the plate incorporation procedure. This is a quantitative test run by combining the test substance with about 10^8 bacteria and rat liver homogenate in 2 ml of molten 0.6% agar containing 0.5 mM histidine and 0.5 mM biotin. The mixture is poured onto the surface of a minimal glucose agar plate and hardens as a thin overlay. Plates are subsequently incubated at 37C for 48 hours. Since the medium contains a limited supply of histidine and biotin, the entire population undergoes only a few divisions before the supply is exhausted and cell division ceases. From this point on, only cells that have reverted or are capable of reverting to histidine independence will grow and produce macroscopic colonies on the plate during the incubation period. These revertant colonies

are scored and their number is compared with the number of spontaneous revertants for the strain being used. If the number of revertants produced by the test substance is twice that of the spontaneous reversion rate, the test is considered positive.

The spot test procedure is a qualitative test. In this modification, test substance is placed on the surface of a minimal glucose agar plate already containing the agar overlay with the test organism and microsomal activation system. The test substance diffuses into the agar setting up a concentration gradient. Revertant colonies surrounding the spotted chemical constitute a positive test. Use of this procedure is limited to compounds which diffuse readily.

The liquid preincubation procedure was developed for use with compounds which do not readily diffuse. In this case, the test solution, microsomal activation system and bacterial culture are combined in sterile tubes and incubated, with shaking, at either 37C for 20 minutes or 30C for 30 minutes. Following this pre-incubation, top agar is added and the tube contents are poured onto the surface of a minimal glucose agar plate, allowed to harden and incubated for 48 hr at 37C before revertants are scored.

Included with the performance of each assay are several controls. Before any substance is tested, certain quality controls are run on the bacterial strains to establish the validity of their special features and also determine the spontaneous reversion rate(1). Since this was the initial use of the Ames assay by this laboratory, historical strain data are not available.

Since the plasmids in strains TA98 and TA100 contain ampicillin resistant markers, we can expose the bacteria to this antibiotic and prove the existence of the plasmid when growth occurs. The removal of the lipopolysaccharide layer allows uptake by the Salmonella of larger molecules. Therefore if a disk previously soaked in crystal violet dye is placed onto a plate containing any of the bacterial strains, a zone of growth inhibition will be evident around the disk. Because the Salmonella can now admit the dye, and since it is toxic to metabolism, the organisms are killed. The absence of excision repair can be determined using ultraviolet (UV) light. Since these repair systems function primarily by excising photodimers between pyrimidine bases, exposure of the bacteria to UV will activate the formation of these dimers and thus cause cell lethality, since repair cannot be made. In order to prove that the bacteria are responsive to the mutation process, positive controls are run with a known potent mutagen. If a large number of revertants are obtained, after exposure to the positive control substance, we are assured of the bacteria's responsiveness.

Sterility controls are also run to determine the presence of contamination and to monitor the density of background lawn growth. Quality control is also confirmed in each of the dilutions used, both activated and nonactivated. The verification of the tester strains occurs spontaneously with the running of each assay. Since the conclusions are based on the spontaneous reversion rate as compared to the chemically induced rate, values are run for this using the same sample of bacteria that is used in the assay. These values are monitored and compared to the values cited by Ames(2). When operating effectively, these strains can detect substances that cause base pair mutations (TA1535, TA100) and frameshift mutations (TA1537, TA1538, and TA98). During the scoring of revertant colonies, lawns on plates are examined microscopically (low power, 100X). Absence of bacterial lawn from a test plate is indicative of a toxic test compound. This suggests that a retesting of the compound at a lower concentration should be conducted. A lawn which is more dense than the negative controls is suggestive of excess histidine present in the system. Finally, colonies scored as revertants are verified as true revertants by transferring them to a minimal glucose agar plate containing no histidine or biotin. Growth on plates following 24 hr incubation at 37C is indicative of true reversion to histidine independence.

Foods can and some foods do contain chemical mutagens. Aflatoxin B_1 may be found in contaminated corn and peanuts. With the Ames assay 100 ng of this toxin per plate generates 1000 histidine revertants in strain TA100(3). This is nearly 7 times the spontaneous reversion rate for this strain. Other mycotoxins have been shown as mutagens by the use of the Ames assay.

Following negative tests for carcinogenicity in rats, furylfuramide (AF2) was used extensively by the Japanese from 1965-1973 as an antibacterial food additive. In 1973, it was found to be highly mutagenic in strains of E. coli, S. typhimurium, yeast and Neurospora. These findings initiated new animal carcinogenicity tests which revealed this compound was a carcinogen. Based on these data, Japanese officials banned the use of AF2 in foods. It is, however, still too soon to tell if the widespread usage of AF2 will increase the cancer rate in Japan(4).

Nitroso compounds are potent carcinogens in laboratory animals and many of these compounds are detected as mutagens in the Ames test(5). The most commonly found preformed nitrosoamine in foods is dimethylnitrosamine which has been identified as a potent carcinogen and can be isolated from hot dogs, ham, bacon, and luncheon meats(6).

EXPERIMENTS

The experiments described in this report were undertaken to assess the application of the Ames Salmonella/mammalian microsome mutagenicity assay for detection of mutagenic potential in thermally processed, frozen, electron-irradiated and gamma-irradiated beef. The beef used in these studies was supplied by the US Army Natick Research and Development Command, Natick, Massachusetts 01760 U.S.A.

Unless specifically stated in this report, all assays were performed as outlined in the following publication: Ames, B.N., J. McCann, and E. Yamasaki, 1975. Method for Detecting Carcinogens and Mutagens with the Salmonella Mammalian Microsome Mutagenicity Test. Mutation Research 31:347-364.

To parallel cooking procedures in normal households, and to avoid bacterial contamination, the meats used in these experiments were prepared as follows: Meat containers were opened aseptically and the contents were transferred to sterile stainless steel pans with foil covers. Slices of meat approximately 1/2 inch thick were cooked for thirty minutes at 170C. Following cooking, meats and their juices were coarsely ground in a sterile, hand-operated meat grinder. The ground meats were combined with sterile water on a 1:1 weight basis and blended for 4 minutes at high speed in a commercial Waring blender. Meats prepared in the foregoing manner were transferred to sterile containers and stored at -20C for use in the following test procedures.

¶TEST NO. 1: Standard Plate Incorporation Test with Top Agar Modifications Using TA100

Meats contain varying quantities of bound and free histidine. This experiment was designed to determine if the free histidine in the four beef products would have any effect on the Ames assay. Using a Durrum 500 amino acid analyzer, we determined the level of free histidine in the meats. The levels of free histidine in the meats were as follows: 33.5 µg/g in frozen beef, 26.7 µg/g thermally processed beef, 30.9 µg/g electron irradiated beef, and 26.9 µg/g gamma-irradiated beef.

METHOD

The plate incorporation assay procedure, using only strain TA100 without microsomal activation, was performed on each of the beef samples. As a result of the free histidine levels contained in each product, the level of histidine supplied in the top agar was reduced from 0.5 mM to 0.43 mM. Additional top agar modifications tested were 0.5 mM biotin, 0.43 mM histidine-0.5 mM biotin, and no addition of either histidine or biotin. Meats were prepared for the

assay by thawing the frozen stock and combining on a 1:1 weight basis with sterile water. This was followed by 3 minutes of high speed blending in a commercial Waring blender. To assure that 0.05 gm meat was used in each assay, 0.2 ml of meat slurry was used in the plate incorporation procedure, instead of the 0.1 ml test chemical specified in the assay directions.

RESULTS AND DISCUSSION

The results of these tests are incorporated in Table 1. Comparison of the average revertant counts in Table 1 with that of the negative control value (363) indicates that none of the meats had any mutagenic potential for strain TA100.

When the amino acid histidine and the vitamin biotin were omitted from the top agar, there was a drastic reduction in the number of revertants as compared to the negative control. This reduction is validation of the requirement for histidine and biotin for the initiation of growth by the revertant levels approximating those of the negative control. This indicates that the meats could supply sufficient histidine for growth initiation.

When only histidine was incorporated in the top agar, revertants of TA100 treated with thermal, gamma or electron-irradiated beef numbered only a third of those when frozen beef was tested. The number of revertants produced by TA100 following treatment with frozen beef approximated those of the control. These results indicate the failure of thermal, electron or gamma-irradiated beef to supply sufficient biotin for growth of the test strain. Apparently frozen beef is capable of meeting the biotin level required by strain TA100.

Combination of histidine and biotin in the top agar yielded revertants after treatment with each of the beef slurries. The numbers of revertants of TA100 treated with frozen, electron and gamma-irradiated beef were less than the negative control, while the number of revertants following treatment with thermally processed beef was slightly higher than the negative control. This number of revertants is, however, not suggestive of mutagenic potential for the level is not the suggested minimum of twice the number of revertants of the negative control.

Table 2 shows the strain control data for TA100 used in this test. The spontaneous revertant levels suggested by the Ames Laboratory are shown in Appendix A. The control procedures performed on strains not used in the assay are found in Appendix B. The revertant counts of the negative controls are significantly higher than the levels stated in Appendix A. This discrepancy was not immediately investigated but, in retrospect it was frequently observed when strain positive and negative controls are not done at the same time as the test. Although the genetic markers of TA100 appeared

normal, its response to the positive control chemicals was not. N-methyl-n'-nitro-n-nitrosogurandine (MNNG) showed no mutagenic activity against TA100 (Table 1). Additionally, when TA100 was combined with microsomal activation, no mutagenicity is seen with 2-aminofluorene (AF), while only slight levels of mutagenicity are seen after treatment with Benzo-α-pyrene (BP) (Appendix B).

¶ TEST NO. 2: Standard Plate Incorporation Procedure with TA100 using a Water Slurry of a Whole Food Item

The plate incorporation procedure was developed primarily to test compounds soluble in one of four diluents: water, dimethyl sulfoxide, p-dioxane or ethanol. Histidine and biotin levels added to the top agar were not reduced in this experiment. This experiment was to examine the performance of a water slurry of a whole food item in the presence of the additional histidine, biotin, and other growth factors supplied by the meat slurries.

METHOD

Slurries of each of the four beef products were prepared as described in Test 1. Using the plate incorporation test procedure, 0.2 ml of each meat slurry was tested against strain TA100 with and without the addition of S-9 (microsomal activation system).

RESULTS AND DISCUSSION

Results for this test are shown in Table 3. The average revertant count of TA100 exceeded that of the negative control by a factor of greater than 2 in all cases except thermally processed beef with activated TA100. The high counts obtained in this experiment may be due to excess histidine in the system. This excess of histidine allows the bacteria to undergo additional cell divisions and appear as small colonies easily mistaken for revertants. This demonstrates the need to confirm these colonies as being true revertants (histidine independent). Results of this experiment must be questioned since the positive controls did not perform as expected with the test strain used. The response of TA100 to MNNG and to AF indicated the strain was not performing as expected. Table 4 indicates that the bacterial strain used was in possession of the genetic markers as were the other four strains (Appendix C).

¶ TEST NO. 3: Standard Plate Incorporation Procedure with all 5 Tester Strains using a Water Slurry of a Whole Food Item

In this experiment, the plate incorporation procedure was used with unaltered histidine and biotin levels in the top agar to test all four beef products against all five tester strains. Revertants produced were verified as true by inoculating on minimal glucose agar, then incubating and examining plates for the presence of growth.

METHOD
Slurries of each meat product were prepared as described in Test No. 1. The incorporation procedure was performed with 0.2 ml of each meat slurry. Plates were prepared with and without the addition of S-9.

RESULTS AND DISCUSSION
Table 5 shows the results of the meats tested and Table 6 displays the results of the experimental controls. Compared with the figures for the negative controls, the numbers of revertants with nonactivated TA1535 was just twice the spontaneous rate for thermally processed beef. The response of TA100 to positive control chemicals AF and MNNG indicated the strain was not performing as expected.

In this test, revertant colonies were tested and verified as true revertants by their subsequent inoculation onto minimal glucose plates without histidine or biotin. Growth on such plates is indicative of the return of the organism to prototrophy and can be used as a quality control check. Due to the difficulty in counting plates, this type of quality assurance procedure should be used when foods are assayed. When we initiated this procedure, we found 9.9% of the colonies tested not to be true revertants (Table 7).

It should be noted here that plates produced with meat slurries are quite difficult to read following the 48 hour incubation period. The problem of discrimination between bacterial colonies and particulate matter arises frequently in food microbiology. These difficulties preclude the use of automated counting equipment, and make the scoring of revertants a tedious and time-consuming process.

TEST NO. 4: Assessment of the Mutagenic Potential of 2,3,5-Triphenyl Tetrazolium Chloride.

Due to its reduction by bacterial action and the formation of red-colored colonies, the chemical 2,3,5-triphenyl tetrazolium chloride is often added to bacteriologic media to facilitate counting colonies amid debris. The plate incorporation test was performed to determine the mutagenic potential of this chemical before incorporating it within the assay system.

METHOD
In an aqueous solution of 20 mg/ml, the compound was tested against the five Ames strains with and without microscomal activation by the plate incorporation procedure.

RESULTS AND DISCUSSION
The results of this test and the controls are presented in Table 8. When compared to the average revertant counts of the negative controls,

2,3,5-triphenyl tetrazolium chloride displays no evidence of mutagenesis. While the test compound showed no evidence of mutagenicity, its counts being lower than those of the negative controls may be suggestive of toxicity. Before being used in the assay system, the compound should be retested at a lower concentration. The response of TA100 to the positive controls, AF and MNNG, was again abnormal.

¶ TEST NO. 5: Standard Plate Incorporation Procedure and Spot Test Procedures Using a Water Slurry of Beef with Known Mutagens Added.

In this experiment, the qualitative and quantitative procedures were performed on the four beef products. Known mutagens were incorporated with each of the beef products tested to determine if the meat slurry was capable of blocking mutagenesis by binding the mutagen and preventing it from entering the bacterial cell.

METHOD

In this test, the qualitative spot and quantitative plate incorporation techniques were used to assay the meat products. In some tests, mutagen was incorporated with the meat. When tester strain TA1535 was used, MNNG without microsomal activation was the mutagen chosen. Microsomal activation and BP were incorporated with the meat and tested against TA1537, TA1538, TA98, and TA100.

RESULTS AND DISCUSSION

The spot test results are shown in Table 9, while results of the plate incorporation tests are in Table 10 and those of the controls in Table 11.

Again it can be seen that MNNG and AF did not induce mutagenesis in strain TA100. Dimethy benzanthracene (DMBA) initiated only a slight level of mutagenesis in TA1538. With regard to the negative controls, the numbers of revertants produced spontaneously by TA98 are considerably lower than the range suggested by Ames et al(2)(Appendix A). Addition of microsomal activation to this strain results in counts two to five times that of unactivated strains. With strain TA100, the number of spontaneous revertants seems to have dropped to approximately two-thirds of the recommended levels. Strain TA1537 shows high levels of revertants without use of activation and when activation is used, the number of spontaneous revertants nearly doubled. Some of the strain markers also show irregularities. Specifically, these are the lack of sensitivity to crystal violet and desoxycholate displayed by TA1537 and TA1538. TA98 also produced an abnormally small zone of sensitivity to desoxycholate.

As seen in Table 9, the spot test yields no hint of mutagenic activity with any of the beef products, but the lawn and revertant pattern were significantly changed. When mutagen was incorporated with the

meats before [illegible] plates, several observations could be made following [illegible] incubation period. No mutagenesis in TA98 was seen when gamma-irradiated beef was combined with benzo (a) pyrene. [illegible] mutagenesis in TA100 was not indicated with either gamma or electron-[illegible] when combined with benzo (a) pyrene. All four beef samples [illegible] mutagenesis with TA1535 although the level of mutagenic activity was lower than that seen in the positive control. [illegible] interaction of thermally processed beef and mutagen showed [illegible] in TA1537. Strain TA1538 showed mutagenic effects in all four beef samples when combined with mutagens. These data indicate some blocking of mutagenic action by the meats.

Photographs have been included in this report to show the lawn and revertant patterns. In Figure 1, strain TA100 was dispersed over the agar plate. Two to three grams of meat were spotted in the center of the plate. As reported earlier, the lawn and the revertant colony pattern were abnormal when meats were spot tested.

The interpretation of the abnormal lawn and revertant colony pattern around the spot is, near near the spot, complex substances have diffused from the meat and were supplying nutrients. Thus, the non-revertant bacterial cells compete with revertant cells for the nutrients contained in the minimal glucose agar and the revertant cells are unable to overgrow the lawn and form macrocolonies. Revertant colonies [illegible] at the border of the zone of dense lawn were quite small because they were competing with the non-[illegible] for nutrients. The revertant colonies increased in [illegible] at greater distances of the plate because there was no competition [illegible] diffusion of meat substances and the start of proper performance [illegible] reaction. Since the area distal to the zone associated [illegible] was greater than the equivalent area on a pour plate, [illegible] with the spotting procedure were lower than those [illegible] with the plate incorporation technique.

[illegible] shows the mutagenic effect of MNNG on TA1535 when MNNG is [illegible] with frozen beef. The spontaneous revertant count for the [illegible] The area of dense lawn surrounding the [illegible] is seen also.

[illegible] shows that gamma-irradiated beef spotted on a plate of [illegible] S. typhimurium results in the formation of a uniform [illegible] of revertant macrocolonies.

[illegible] incorporation procedure using strain [illegible] beef, the number of revertants was [illegible] spontaneous revertant count. Par-[illegible] the meat product prevents usage of [illegible] count. It also makes plates difficult to [illegible]

Figure 5 shows revertant colonies formed by TA1538 when mutagen was combined with thermally processed beef and set up by the plate incorporation technique. The numbers of revertants scored were within the range of the number of revertants scored when mutagen is tested alone.

Figure 6 demonstrates no revertant colonies appearing when a wild type or non-mutated strain of Salmonella typhimurium is used with beef in the plate incorporation technique.

The results of the plate incorporation tests, Table 10, of meats without mutagen were suggestive of mutagenesis with all four beef products tested against strains TA98, TA100, and TA1535. Levels of mutagenesis were increased when meat was combined with mutagen. It should be noted that no verification of revertants was done and that the strains were not responding as expected during the control procedures.

¶ TEST NO. 6: Pre-incubation of Tester Strains with Water Extract from Meats Processed by the Four Different Methods.

Some mutagens cannot be efficiently detected by using the standard plate incorporation test. They can, however, be tested by using a modification of the test. The modification, called the liquid pre-incubation assay, calls for the chemical being tested to be incubated with activated liver microsome preparation (S-9 mix) and the bacterial culture before incorporation into the top agar overlay.

In this experiment, extracts from each of the four beef products were pre-incubated with each of the five tester strains before the plate incorporation assay.

METHOD

Meat extracts were prepared by blending a 1 part meat and 1 part water slurry for 3 minutes in a high speed blender. The slurries were then centrifuged at 8,000 g for 45 minutes at 4C. The supernatant was removed and referred to as meat extracts.

Test strains were grown in a nutrient broth for 16 hours at before being removed from their growth medium by centrifugation for 45 minutes at 4C, 2,700 g. The supernatant was discarded and the packed cells were resuspended in the original volume of 0.9% saline. One milliliter of the suspension was withdrawn and plated for a determination of the initial viable cell population. The rest of each suspension was combined in each of three following combinations:

A. 3 cc cell suspension + 3 cc meat extract.
B. 3 cc cell suspension + 3 cc meat extract + 15 cc S-9 mixture.
C. 3 cc cell suspension + 3 cc 0.9% saline (control).

The above combinations were incubated for 2 hours at 37C. Following incubation, the viable cell count was again determined. Cells were washed twice and resuspended in saline. A final population determination was made following washing and the remainder of the suspension was used for an Ames assay by the plate incorporation method.

RESULTS AND DISCUSSION

Results from this experiment are reported in Tables 12-16. Following the completion of this test with its aberrant results, the meat slurries were examined and all were contaminated with bacteria other than the tester strains. This finding invalidates the experimental data and illustrates the fact that S. typhimurium is not the only bacterium capable of initiating growth on minimal glucose agar.

TEST NO. 7: Standard Plate Incorporation Procedure with Top Agar Modification Using all 5 Tester Strains.

This experiment was a repeat of Test 1 in which the levels of histidine supplied to the bacterial strain in the top agar were reduced corresponding to the amount of free histidine contained in the meat product.

METHOD

A slurry of each beef product was prepared and 0.2 ml of the slurry was tested by the plate incorporation procedure using 0.43 mM histidine and 0.5 mM biotin in the top agar.

RESULTS AND DISCUSSION

Tables [illegible] and 16 show the results of the tests and controls in this experiment. The positive revertant rate was higher than recommended by Ames et al(2) for TA100 and TA1535. Strain TA100 demonstrated no mutagenesis when tested with MNNG or BP. TA1538 showed no mutagenesis when treated with DMBA.

Three meats appeared to exhibit toxicity for the test strains. This is evidenced by the low levels of revertants scored with thermal, freeze, and electron-irradiated beef and strains TA98, TA1535, TA1537, and TA1538. Gamma-irradiated beef showed the opposite, revertant levels were indicative of mutagenesis with all strains except TA100 without microsomal activation. However, there were two distinct types of colonies on the plates. Biochemical identification completed on the 2 types of organisms revealed one type to be S. typhimurium and the other to be Klebsiella pneumoniae. These findings negated any hint of mutagenesis; however, they do emphasize the need for not only checks to prove that revertants are valid, but also the additional verification of the identity of the organism.

When revertants were picked to minimal glucose agar plates containing no histidine or biotin, the majority of revertants grew on the medium (Table 18), except for those revertants picked from plates where gamma-irradiated beef was tested with activation. In this case, less than 50% of the revertants could be called true revertants. The results of these tests were invalid and no conclusions can be drawn.

¶ TEST NO. 8: The Effect of Varying the Histidine and Biotin Concentrations in the Top Agar when Testing Thermal, Frozen, Electron-Irradiated and Gamma-Irradiated Beef.

This experiment was designed to demonstrate the effects of varying the histidine and biotin concentrations in the top agar on the performance of the plate incorporation assay on beef preserved by thermal processing, freezing, or irradiation by electron or gamma rays.

A new batch of cooked meat samples was prepared. Containers of thermal, frozen, electron-irradiated, and gamma-irradiated beef were opened aseptically and emptied into sterile pans. Meats were cooked at 170C for 30 minutes. Following cooking, meats and their juices were coarsely ground in a sterile hand operated meat grinder. Coarsely ground meats were combined with sterile water on a 1:1 basis by weight and blended for four minutes at high speed in a commercial Waring blender. Meats were transferred to sterile containers and stored at -20C for use in this and subsequent experiments.

METHOD

A portion of each of the meats prepared above was thawed and combined on a 1:1 weight basis with water followed by 3 minutes of high speed blending. Of the slurry prepared from each meat, 0.2 ml was used as the test compound in each plate incorporation Ames assay. The top agar used in performance of these assays

1. 0.43 mM histidine
2. 0.5 mM biotin
3. 0.43 mM histidine + 0.5 mM biotin
4. No additives

RESULTS AND DISCUSSION

The results of these assays are shown in Tables 19-23. When the bacterial test strains were supplied with only a source of histidine, revertants were not seen on the plates (Table 19). This is considered as validation of the biotin requirement for growth of these organisms. It is also indicative of the meats inability to provide a sufficient quantity of this vitamin. The results seen in Table 20 indicate that if biotin is supplied, the meats contributed sufficient quantity of histidine to initiate cell growth and revertant formation. The

revertant levels seen in this table are for the most part not suggestive of mutagenesis. Those showing revertant levels suggestive of mutagenesis are thermally processed beef, frozen beef, and gamma irradiated beef when tested against nonactivated TA98 and gamma irradiated beef tested with activation against TA1537. A maximum of six revertants from each plate was picked and all were confirmed as true revertants. The revertants scored on plates when provided with both histidine and biotin are seen in Table 21. Meats showed no evidence of mutagenesis, however, the fact that the numbers of revertants were less than those of the negative controls, may be indicative of toxicity or of the meats supplying complex growth factors that mask the response because of increased non-revertant growth. All the test strains used in these assays contained a mutation to allow increased permeability of the cell wall by producing a defective lipopolysaccharide layer. It is possible that with this mutation some compound in the meat, not normally able to penetrate the cell wall, was able to enter the cell and produce death rather than a bacterial mutation.

Table 22 shows, as expected, no revertants without an exogenous source of histidine and biotin in the medium. The positive controls showed no mutagenesis for TA100 with AF or MNNG; and they showed no mutagenesis for TA1538 with AF or DMBA.

TEST NO. 9: Preincubation of Tester Strains with Meat Extract from Meats Processed by Heating, Freezing, and Irradiating with Electron and Gamma Rays.

This series of assays was an attempt at performing the liquid preincubation procedure with slurries of the four types of preserved beef, similar to Test No. 6.

METHODS

The methods used in this test were the same as those reported earlier in this paper for Test No. 6.

RESULTS AND DISCUSSION

Tables 24-29 show the results for these assays and show considerable difficulties encountered in performance of the liquid preincubation assay procedure.

Table 24 indicates that although a sufficient cell population was available for test performance, the bacterial lawns on the assay plates were abnormal, thus resulting in an uncountable set of plates.

In Table 25 one can see what appears to be high numbers of revertants; however, the cell population was only about 10^7, a figure far lower than that recommended for the procedure. When fewer cells are present, there is more available histidine for each cell, hence the background lawn undergoes more than just a few generations and the distinction

Again in Table 26 the cell population is lower than the recommended 10^8 cells. The revertant levels seen are, however, within the normal range for this strain and are, therefore, not indicative of mutagenesis. The results of testing the four beef products against strain TA1537 are presented in Table 27. The final cell population was sufficient for the assay, yet no lawns were seen on many of the test plates. The same situation existed for strain TA1538 as seen in Table 28. No explanation for this observation was apparent.

TEST NO. 10: Preincubation of Tester Strains with Water Extracts of Thermally Processed and Gamma-Irradiated Beef.

The plates from the preincubated meat slurries were hard to count. Thus, instead of testing slurries of the meats, this experiment was designed to test water extracts of the thermally processed and gamma-irradiated beef products.

METHOD

Meat slurries were prepared from a 1:1 combination of each frozen meat stock with sterile water. Following 3 minutes of blending at high speed, the slurries were centrifuged for 45 minutes at 4C, 8,000 g. The supernatant was tested by the plate incorporation procedure with altered levels of top agar additives.

RESULTS AND DISCUSSION

Results for these assays and the appropriate controls are seen in Tables 29-30. As can be seen in the table, if histidine at concentrations of either 0.43 mM or 0.5 mM is all that is supplied, true revertants are not generated. When biotin was the only additive supplied, many revertants were generated by both thermally processed beef and gamma-irradiated beef in TA98, TA100 and TA1538 the levels of which were suggestive of mutagenesis.

Revertants produced by strains tested when supplied histidine and biotin were about the levels of the negative controls. With respect to the positive controls, TA100 exhibited no mutagenesis with AF or BP which is suggestive of loss of enzyme activity in the S-9 preparation.

TEST NO. 11: Preincubation of Tester Strains with Water Extract of Frozen and Electron Irradiated Beef.

This series of plate incorporation assays was to test water extracts of frozen and electron-irradiated beef.

METHOD

A water extract was prepared from each beef slurry and used in the plate incorporation assay procedure, as described in Test No. 10.

RESULTS AND DISCUSSION

Table 31 displays the results of these assays. Table 31 shows that unless biotin is available, no cell growth took place and no revertants are produced. When biotin alone is supplied with meat slurries, revertants were produced indicating that the meats supply some histidine; the level of revertants with biotin alone, was unpredictable as the revertant level varied from lower to higher than expected. The levels of revertants produced when histidine and biotin are supplied were quite high in number, especially with strain TA100. Controls are shown in Table 32.

TEST NO. 12: Preincubation of Tester Strains with Water Extracts of Thermally Processed and Gamma-Irradiated Beef.

This series of plate incorporation assays was to test water extracts of thermally processed and gamma-irradiated beef.

METHOD

Water extracts were prepared from slurries of the meats as previously described in Test No. 10.

RESULTS AND DISCUSSION

The results of positive and strain control tests in Table 33 indicate an apparent failure of the S-9 mix enzyme preparation. The strain markers appeared to be intact although TA98 displayed a small zone of inhibition to crystal violet indicating a loss of sensitivity. Due to the failure of the positive controls, the data in Table 34 cannot be considered definitive, but appear to be consistent with those obtained in Tests 10 and 11.

TEST 13: The Plate Incorporation Procedure Used to Test the Water Slurries of Four Beef Products.

With the uncertainty of the use of the proper S-9 concentration in the past, it was decided to go back and repeat some of the experiments done earlier in this study. This experiment was designed to examine the performance of the plate incorporation procedure on a water slurry prepared from a whole food item containing free histidine.

METHODS

The plate incorporation test procedure was performed using a water slurry of each beef product. Concentration of top agar additives, histidine or biotin, were varied as follows:

1. 0.5 mM histidine.
2. 0.43 mM histidine + 0.5 mM biotin.
3. 0.5 mM biotin.
4. 0.43 mM histidine.

RESULTS AND DISCUSSION

Tables 35-40 show the results for this series of assays. A supply of only histidine, regardless of amount, was insufficient for growth initiation of the test strains used in this assay (Tables 35 and 38).

Table 36 shows that 0.43 mM histidine + 0.5 mM biotin is sufficient for growth initiation and the production of revertants. Of the revertant levels seen here, those of strains TA100 and TA1535 showed mutagenesis with all four beef products. Table 40 shows the rate of true revertants. The reason for the high level of false positives in this test is unexplained but it caused the validity of the data to be questioned.

When only biotin was supplied to the strains, revertants were still produced, although their numbers were somewhat reduced. In Table 37, counts indicative of mutagenesis are again seen with TA100 and TA1535, although they are only seen in thermally processed and frozen beef. These results were again questioned because of the high level of false positives.

¶TEST 14: The Plate Incorporation Procedure Used to Test the Water Extracts of Four Beef Products.

METHOD

Water extracts of the beef products were prepared as described in Test 6.

In Table 41, the results of plate incorporation tests using beef slurries with 0.5 mM histidine + 0.5 mM biotin in the top agar can be seen. As before, revertant count levels of TA100 and TA1535 are suggestive of mutagenesis with all four beef products. Since these plates had excess histidine, apparent revertants may not have been more than overgrown lawn in light of the high levels of false positives previously reported.

Table 42 shows the results of plate incorporation assays with water extracts of the meats. These results reveal that counts for TA1535 are indicative of mutagenesis with all four beef products as are those for TA1538 and some of the counts for TA98 and TA100.

The controls in Table 43 show the strains to be in possession of their markers and the positive controls to induce mutagenesis. Although the level of revertants of TA100 with AF is low, this level is appropriate for the mixture used. The results of this experiment point out the need to verify a representative portion of revertants at random. However, no verification of revertants was done.

TEST 15: The Plate Incorporation Procedure Used to Test Water Extracts of Beef Processed Four Different Ways.

This experiment was designed to determine if the apparent mutagenesis seen with the water extracts of the four beef products could be validated.

METHOD

The plate incorporation assays were performed on water extracts of the meats prepared by centrifuging the slurries and testing the supernatant.

RESULTS AND DISCUSSION

Tables 44 and 45 show the results and indicate mutagenesis in TA100, TA1535, TA1538, and TA98. These results should be questioned due to the fact revertants were not verified. In addition, spontaneous revertant levels seen in Table 45, which indicate TA100 was producing only half the number of revertants in the range suggested by Ames et al(2).

CONCLUSIONS

Considerable difficulties were encountered in the performance of the Ames Assay with a whole food item. Meats contain histidine as well as other chemical complexes which interfere with the test. Consequently, the Ames Assay was inappropriately applied to the meat items, and that the resulting data are of limited value in assessing the mutagenic potential of meat items. Nonetheless, it is also our opinion that the tests conducted do not indicate mutagenicity of any of the meat products or processes.

RECOMMENDATIONS

Recommend complex compounds, such as meats, be fractionated and the individual fractions be tested so that complications resulting from excess histidine and other growth factors can be eliminated.

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LIST OF APPENDICES

APPENDICES

Suggested Spontaneous Revertant Level

APPENDIX A

Appendix A. Suggested spontaneous revertant level

Strain	Average	Range
TA1535	20	10-35
TA1537	7	3-15
TA1538	25	15-35
TA100	160	120-200
TA98	40	30-50

SOURCE: Reference 2, Method for Detecting Carcinogens and Mutagens with the Salmonella/mammalian Microsome, by Ames et al.

Supplemental Information for Test 1

APPENDIX B

Appendix B. Supplemental information for Test 1

1. Strain control for test strains not used in Test 1

Strain	Histidine Requirement	Ampicillin Resistance	Sensitivity to UV	Crystal Violet	Desoxycholate	Spontaneous Revertants
1535	+	NT	+	13 mm	17 mm	11
1537	+	-	+	16 mm	20 mm	16
1538	+	NT	+	19 mm	20 mm	17
98	+	+	+	16 mm	17 mm	42
WT	-	NT	-	8 mm	9 mm	N/A

2. Positive controls for tester strains and treatments not used in Test 1

Strain		98A	1535	1537A	1538A	100A
Control	Chemical					
AF	2 µg/plate	284,283 (284)			281,326 (304)	439,439 (439)
MNNG	20 µg/plate		3181,3089 (3135)			
DMBA	20 µg/plate	347,345 (346)		196,138 (167)		1833,1716 (1774)
BP	2 µg/plate	511,743 (627)		196,189 (192)	322,287 (304)	988,979 (984)

- = no S-9 or negative response (as appropriate)
+ = S-9 or positive response (as appropriate)
() = average
A = Activation with S-9

Appendix B. Supplemental information for Test 1 (Cont'd)

3. Negative controls for tester strains not used in Test 1

Strain	S-9	98	1535	1537	1538
Count	-	58,73 (66)	85,74 (80)	24,26 (25)	48,28 (38)
	+	81,75 (78)	145,143 (144)	23,25 (24)	55,69 (62)

- = no S-9 or negative response (as appropriate)
+ = S-9 or positive response (as appropriate)
() = average

Supplemental Information for Test 2

APPENDIX C

Appendix C. Supplemental information for Test 2

1. Strain control for tester strains not used in Test 2

Strain	Histidine Requirement	Ampicillin Resistance	Sensitivity to UV	Crystal Violet	Desoxycholate	Spontaneous Revertants
1535	+	NT	+	14 mm	15 mm	20
1537	+	-	+	18 mm	30 mm	17
1538	+	NT	+	21 mm	29 mm	14
98	+	+	+	15 mm	17 mm	37
WT	-	NT	-	NT	NT	N/A

2. Positive controls for tester strains not used in Test 2

Strain	98A	1535	1537A	1538A
Control chemical				
AF 2 µg/plate	254,256 (255)			214,184 (199)
MNNG 20 µg/plate		1296,834 (1065)		
DMBA 20 µg/plate	170,246 (208)		55,36 (46)	
BP 2 µg/plate	312,264 (288)		77,72 (74)	85,127 (106)

- = no S-9 added or negative response (as appropriate)
+ = S-9 added or positive response (as appropriate)
() = average

Appendix C. Supplemental information for Test 2 (Cont'd)

3. Negative control for tester strains not used in Test 2

Strain	S-9	98	1535	1537	1538
Count	-	25,29 (27)	16,12 (14)	4,9 (6)	12,9 (10)
	+	61,58 (60)	11,22 (16)	9,6 (8)	40,33 (36)

- = no S-9 added or negative response (as appropriate)
+ = S-9 added or positive response (as appropriate)
() = average

TABLES

APPENDIX D

LIST OF TABLES

LIST OF TABLES, CONTINUED

LIST OF TABLES, CONTINUED

TABLE 1. Plate Incorporation Test of Beef Using Strain TA100 with Altered Levels of Histidine and Biotin in Top Agar *

Test Material	Amount of Test Material Added	S-9 Mix Added[a]	Revertant Count per Plate with Top Agar Modification: 0.43mM Histidine	0.5mM Biotin	0.43mM Histidine-0.5mM Biotin	no Histidine or Biotin	0.5mM histidine-0.5mM Biotin
MNNG	2 µg/0.1ml	-					588,614 (601)[d]
None	N/A	-					370,356 (363)
FB	0.2 ml	-	363,300 (332)	480,450 (465)	200,200 (200)	0,1 (1)	NT[b]
TPB	0.2 ml		80,150 (115)	382,464 (423)	754,408 (581)	2,0 (1)	NT
GIB	0.2 ml	-	150,LA[c] (150)	456,450 (453)	200,150 (175)	1,2 (2)	NT
EIB	0.2 ml	-	100,100 (100)	322,350 (336)	160,250 (205)	2,1 (2)	NT

[a] + = 0.5 ml S-9 mixture added, - = no S-9 mixture added

[b] NT = not tested

[c] LA = laboratory accident

[d] () = average of revertant counts on two plates

* Some counts estimated due to counting difficulty

TABLE 2. Strain Quality Control for Test 1

Strain	Histidine Requirement	Ampicillin Resistance	UV	Sensitivity to Crystal Violet	Sensitivity to Desoxycholate	Spontaneous Revertants
TA100	+	+	+	15 mm	16 mm	186

+ = required or positive

TABLE 3. Plate Incorporation Test of Beef Using Strain TA100 with and without Microsomal Activation

Test Material	Amount of Test Material Added	S-9 mix added[a]	Revertant Count per Plate	
MNNG	2 µg/0.1 ml	-	218,249	(234)
AF	2 µg/0.1 ml	+	298,232	(265)
DMBA	20 µg/0.1 ml	+	886,958	(922)
BP	2 µg/0.1 ml	+	523,617	(570)
None	N/A	-	210,176	(193)
		+	212,220	(216)
TPB	0.2 ml	+	363,420	(392)
		-	406,485	(446)
FB	0.2 ml	+	805,881	(843)
		-	652,504	(578)
GIB	0.2 ml	+	656,649	(652)
		-	444,556	(500)
EIB	0.2 ml	+	579,698	(638)
		-	561,426	(494)

a + = 0.5 ml S-9 mixture added
- = no mixture added

() = average of replicate plates

TABLE 4. Quality Control for Strain TA100 used in Test 2

	Histidine Requirement	Ampicillin Resistance	UV	Sensitivity to		Spontaneous Revertants
				Crystal Violet	Desoxycholate	
TA100	+	+	+	14mm	14mm	186

TABLE 5. Plate Incorporation Test of Beef using 5 Tester Strains with and without Microsomal Activation

Test Material	Amount of Test Material Added	S-9 Mix Added[a]	Revertant Count/Plate in Tester Strains				
			98	100	1535	1537	1538
AF	2 µg/0.1 ml	+	245,308 (276)	294,332 (313)			342,352 (347)
MNNG	2 µg/0.1 ml	-		280,306 (293)			
MNNG	20 µg/0.1 ml	-			1433,1502 (1468)		
DMBA	20 µg/0.1 ml	+	206,216 (211)	1106,919 (1012)		95,70 (82)	
BP	2 µg/0.1 ml	+	357,302 (330)	666,667 (666)		152,181 (166)	189,215 (202)
None	N/A	+	25,28 (26)	217,191 (204)	16,13 (14)	15,11 (13)	24,14 (19)
None	N/A		56,48 (52)	218,239 (228)	16,19 (18)	13,18 (16)	36,42 (39)
FB	0.2 ml	+	36,44,55 (45)	252,216,157 (208)	5,16,0 (7)	3,4,4 (4)	0,2,4 (2)
TPB	0.2 ml	+	20,23,39 (27)	366,349,248 (321)	10,28,5 (14)	2,2,7 (4)	17,24,5 (15)
GIB	0.2 ml	+	9,1,0 (3)	272,171,277 (240)	44,43,17 (35)	0,0,2 (1)	0,0,5 (2)
EIB	0.2 ml	+	37,42,51 (43)	323,391,391 (368)	28,15,29 (24)	0,3,6 (3)	14,11,12 (12)

TABLE 5. Plate Incorporation Test of Beef using 5 Tester Strains with and without Microsomal Activation (Cont'd)

Test Material	Amount of Test Material Added	S-9 Mix Added[a]	Revertant Count/Plate in Tester Strains				
			98	100	1535	1537	1538
FB	0.2 ml	-	0,0,21 (7)	291,403,304 (333)	19,7,66 (31)	2,2,4 (3)	0,2,1 (1)
TPB	0.2 ml	-	47,19,18 (28)	334,206,351 (297)	37,47,26 (37)	1,2,0 (1)	35,3,14 (17)
GIB	0.2 ml	-	2,2,8 (4)	355,262,218 (278)	27,24,38 (30)	1,0,0 (0)	0,0,0 (0)
EIB	0.2 ml	-	21,7,LA (14)	182,318,354 (285)	51,10,34 (32)	0,1,1 (1)	2,3,1 (2)

a + = 0.5 ml S-9 mix (microsomal prep) added
- = no microsomal activation

() = average of replicate plates

TABLE 6. Quality Control for Tester Strains Used in Test 3

Strains	Histidine Requirement	Ampicillin Resistance	Sensitivity to UV	Sensitivity to Crystal Violet	Sensitivity to Desoxycholate	Spontaneous Revertants
1535	+	NT	+	14mm	16mm	18
1537	+	-	+	21mm	26mm	17
1538	+	NT	+	21mm	25mm	14
98	+	+	+	18mm	18mm	67
100	+	+	+	16mm	18mm	239
WT	-	NT	-	-	-	N/A

NT = Not Tested
WT = Wild Type Salmonella typhimurium

TABLE 7. Verification of Revertants Produced in Test 3

Test Material	S-9 Mix Added[a]	Strains: True Revertants/Apparent Revertants Tested				
		98	100	1535	1537	1538
None	+	6/6	18/20	4/4	4/4	3/4
	-	3/3	21/23	3/4	3/4	4/4
FB	+	3/3	20/20	2/6	3/4	1/2
TPB	+	5/6	18/18	4/5	2/2	3/3
GIB	+	1/2	18/18	5/6	1/1	2/3
EIB	+	6/6	18/18	3/3	2/2	4/4
FB	-	5/5	20/20	4/6	0/2	1/1
TBP	-	3/6	18/18	6/6	2/2	0/4
GIB	-	5/5	18/18	5/6	0/1	NT
EIB	-	3/3	18/18	6/6	2/2	3/4

a + = S-9 mixture added to original test

- = S-9 mixture not added to original test

TABLE 8. **Mutagenesis Test of 2,3,5 Triphenyl Tetrazolium Chloride**

Strain Quality Control

Strains	Histidine Requirement	Ampicillin Resistance	Sensitivity to UV	Sensitivity to Crystal Violet	Sensitivity to Desoxycholate	Spontaneous Revertants
1535	+	NT	+	15 mm	16 mm	13
1537	+	-	+	20 mm	No Zone	17
1538	+	NT	+	19 mm	No Zone	21
98	+	+	+	16 mm	19 mm	42
100	+	+	+	16 mm	17 mm	159
WT	-	NT	-	No Zone	10 mm	N/A

Positive Controls		Strains 98A	100	100A	1535	1537A	1538A
AF	2µg/0.1 ml	402,353 (378)		281,234 (258)			308,308 (308)
MNNG	2µg/0.1 ml		300,339 (320)				
MNNG	20µg 0.1 ml				690,371 **(530)**		
DMBA	20µg/0.1 ml	186,218 (202)		717,697 (707)		55,43 (49)	32,30 (31)
BP	2µg/0.1 ml	421,333 (377)		571,526 **(548)**		157,130 (144)	194,190 (192)

A = indicates microsomal activation

Table 8 (Continued)

Test Sub. Added	Quantity	S-9 Mix Added	Strain				
			98	100	[illegible]	1537	1538
None		-	99,87 (93)	265,255 (250)	11,10 (10)	9,12 (10)	12,16 (14)
		+	162,174 (168)	245,213 (229)	20,13 (16)	6,16 (11)	41,63 (52)
2,3,5 Triphenyl Tetrazolium Chloride	20mg/ml	-	83,91 (87)	167,185 (176)	4,2 (3)	6,13 (10)	11,13 (12)
	20mg/ml	+	102,144 (123)	116,165 (140)	16,20 (18)	10,11 (10)	18,21 (20)

A = indicates microsomal activation

TABLE 9 - Spot Test of Beef and Beef Seeded with Known Mutagens

Test Material	Amount of Test Material Added	S-9 Mix Added	Revertant Count Per Plate (Avg.) Strains 98	100	1535	1537	1538
None	N/A	+	59,52 (56)	108,102 (105)	10,19 (14)	54,49 (52)	34,39 (36)
None	N/A	-	10,21 (16)	105,107 (106)	16,14 (15)	24,29 (26)	16,14 (15)
TPB	0.2 ml	-	13,15 (14)	207,181 (194)	10,8 (9)	35,25 (30)	3,10 (6)
FB	0.2 ml	-	14,26 (20)	104,136 (120)	15,13 (14)	26,26 (26)	10,6 (8)
GIB	0.2 ml	-	17,18 (18)	117,130 (124)	10,10 (10)	30,20 (25)	3,5 (4)
EIB	0.2 ml	-	20,14 (17)	133,116 (124)	9,10 (10)	25,25 (25)	9,10 (10)
TPB + BP	2 µg/0.1 ml	+	107,129 (118)	198,223 (210)	-	99,118 (108)	103,95 (99)
TPB + MNNG	20 µg/0.1 ml	-	-	-	682,614 (648)	-	-
FB + BP	2 µg/0.1 ml	+	112,178 (145)	243,159 (201)	-	82,LA (82)	121,110 (116)
FB + MNNG	20 µg/0.1 ml	-	-	-	652,LA (652)	-	-
GIB + BP	2 µg/0.1 ml	+	61,77 (69)	108,207 (158)	-	83,58 (70)	92,64 (78)
GIB + MNNG	20 µg/0.1 ml	-	-	-	287,271 (279)	-	-
EIB + BP	2 µg/0.1 ml	+	97,109 (103)	183,130 (156)	-	62,83 (72)	136,113 (124)
EIB + MNNG	20 µg/0.1 ml	-	-	-	463,394 (428)	-	-

TABLE 10 - Plate Incorporation Test of Beer and Beef Seeded With Known Mutagens

Test Material	Amount of Test Material Added	S-9 Mix Added	Revertant Count Per Plate (Avg.) Strains				
			98	100	1535	1537	1538
None	N/A	+	59,52 (56)	108,102 (105)	10,19 (14)	54,49 (52)	34,39 (36)
None	N/A	-	10,21 (16)	105,107 (106)	16,14 (15)	29,24 (26)	16,14 (15)
TPB	0.2 ml	-	34,63 (48)	371,353 (362)	83,54 (68)	7,7 (7)	50,29 (40)
FB	0.2 ml	-	48,96 (72)	444,395 (420)	41,59 (50)	11,7 (9)	30,21 (26)
GIB	0.2 ml	-	34,43 (38)	332,353 (342)	45,55 (50)	13,13 (13)	32,21 (26)
EIB	0.2 ml	-	47,48 (48)	348,356 (352)	43,47 (45)	17,12 (14)	27,32 (30)
TPB + BP	2 ug/0.1 ml	+	192,172 (182)	509,382 (446)	-	43,38 (40)	169,340 (254)
TPB + MNNG	20 ug/0.1 ml	-	-	-	1184,992 (1088)	-	-
FB + PB	2 ug/0.1 ml	+	160,105 (132)	409,416 (412)	-	37,18 (28)	112,132 (122)
FB + MNNG	20 ug/0.1 ml	-	-	-	1241,1379 (1310)	-	-
GIB + BP	2 ug/0.1 ml	+	192,172 (182)	841,766 (804)	-	38,30 (34)	104,170 (137)
GIB + MNNG	20 ug/0.1 ml	-	-	-	1325,1478 (1402)	-	-
EIB + BP	2 ug/0.1 ml	+	255,224 (240)	450,565 (508)	-	40,50 (45)	192,120 (44)
EIB + MNNG	20 ug/0.1 ml	-	-	-	1952,1973 (1962)	-	-

TABLE 11. Test Number 5, Standard Plate Incorporation and Spot Test Procedure Using a Water Slurry of Beef with Known Mutagens Added

Strain Quality Control

Strains	Histidine Requirement	Ampicillin Resistance	Sensitivity to UV	Sensitivity to Crystal Violet	Sensitivity to Desoxycholate	Spontaneous Revertants
1535	+	NT	+	18 mm	19 mm	9
1537	+	-	+	No Zone	No Zone	15
1538	+	NT	+	No Zone	No Zone	16
98	+	+	+	15 mm	9 mm	25
100	+	+	+	19 mm	20 mm	157
WT	-	NT	-	-	12 mm	N/A

Positive Controls

Compound	Amount Tested	S-9 Mix Added	Strain No.. 98	100	1535	1537	1538
AF	2µg/0.1 ml	+	184,265 (224)	147,148 (148)			250,271 (260)
MNNG	2µg/0.1 ml	-		242,210 (226)			
MNNG	20µg/0.1 ml	-			1220,1927 (1574)		
DMBA	20µg/0.1 ml	+	164,184 (174)	649,497 (573)		94,116 (105)	37,52 (44)
BP	2µg/0.1 ml	+	179,211 (195)	383,345 (364)		159,191 (175)	115,141 (128)

TABLE 12. Preincubation Test of Beef using Strain TA 1535

	Saline Control		Beef TPB		Beef FB		Beef GIB		Beef EIB	
	X	A	X	A	X	A	X	A	X	A
Plate Incorporation Test (Revertants/plate)	1307 676 695	982 770 350	279 269 174	285 200 174	89 111 57	149 219 142	180 277 244	206 338 299	118 238 175	177 154 261
Revertant Average	893	701	241	220	86	170	234	281	177	197
Initial Viable Cell Count	$\frac{169}{191}$ / 1.80×10^5		$\frac{139}{144}$ / 1.42×10^5		$\frac{152}{133}$ / 1.42×10^5		$\frac{158}{121}$ / 1.40×10^5		$\frac{106}{106}$ / 1.06×10^5	
Cell count following 2 hr incubation with beef	$\frac{188}{241}$ / 2.14×10^5	$\frac{99}{112}$ / 1.06×10^5	$\frac{119}{133}$ / 1.26×10^5	$\frac{67}{77}$ / 7.2×10^4	$\frac{123}{153}$ / 1.38×10^5	$\frac{57}{70}$ / 6.4×10^4	$\frac{34}{39}$ / 3.6×10^5	$\frac{75}{73}$ / 7.4×10^4	$\frac{164}{147}$ / 1.56×10^5	$\frac{71}{54}$ / 6.2×10^4
Cell count following washing	$\frac{107}{86}$ / 9.6×10^4	$\frac{30}{17}$ / 2.4×10^5	$\frac{137}{125}$ / 1.31×10^5	$\frac{66}{70}$ / 6.8×10^4	$\frac{177}{129}$ / 1.53×10^5	$\frac{57}{116}$ / 8.6×10^4	$\frac{111}{72}$ / 9.2×10^4	$\frac{57}{69}$ / 6.3×10^4	$\frac{98}{112}$ / 1.05×10^5	$\frac{155}{59}$ / 1.07×10^5

X = No S-9 mix added
A = 0.5 S-9 mix added

TABLE 13. Preincubation Test of Beef using Strain TA 1537

	Beef									
	Saline Control		TPB		FB		GIB		EIB	
	X	A	X	A	X	A	X	A	X	A
Plate Incorporation Test (Revertants/plate)	18 18 15	33 47 43	5 4 8	34 33 29	73 121 75	30 25 25	110 71 74	37 37 36	22 13 7	101 64 68
Revertant Average	17	41	6	32	90	27	85	37	14	78
Initial Viable Cell Count	$\frac{94}{89}$ 9.2×10^8		$\frac{68}{75}$ 7.2×10^8		$\frac{53}{34}$ 4.4×10^8		$\frac{59}{72}$ 6.6×10^8		$\frac{76}{68}$ 7.2×10^8	
Cell count following 2 hr incubation with beef	$\frac{113}{106}$ 1.10×10^8	$\frac{131}{116}$ 1.24×10^8	$\frac{37}{37}$ 3.7×10^8	$\frac{139}{121}$ 1.3×10^8	$\frac{194}{259}$ 2.26×10^8	$\frac{119}{123}$ 1.21×10^8	$\frac{307}{328}$ 3.18×10^8	$\frac{290}{187}$ 2.38×10^8	$\frac{269}{256}$ 2.62×10^8	$\frac{112}{114}$ 1.13×10^8
Cell count following washing	$\frac{65}{58}$ 6.2×10^7	$\frac{54}{57}$ 5.6×10^7	$\frac{185}{191}$ 1.88×10^8	$\frac{59}{69}$ 6.4×10^7	$\frac{94}{78}$ 8.6×10^7	$\frac{\cdot\cdot}{45}$ 4.5×10^7	$\frac{122}{124}$ 1.23×10^8	$\frac{80}{73}$ 7.6×10^7	$\frac{102}{106}$ 1.04×10^8	$\frac{37}{47}$ 4.2×10^7

X = No S-9 mix added
A = 0.5 S-9 mix added

TABLE 14. Preincubation Test of Beef using Strain TA 1538

	Saline Control		Beef							
			TPB		FB		GIB		EIB	
	X	A	X	A	X	A	X	A	X	A
Plate Incorporation Test (Revertants/plate)	6	15	20	18	4	15	42	25	73	20
	10	28	27	6	6	14	38	68	96	20
	0	26	13	9	5	8	30	44	11	20
Revertant Average	5	23	20	11	5	12	37	46	60	20
Initial Viable Cell Count	TNTC		$\frac{98}{84}{}_{9.1\times10^{8}}$		$\frac{45}{72}{}_{5.8\times10^{8}}$		$\frac{79}{57}{}_{6.8\times10^{8}}$		$\frac{76}{76}{}_{7.6\times10^{8}}$	
Cell count following 2 hr incubation with beef	$\frac{50}{47}{}_{4.8\times10^{7}}$	$\frac{123}{119}{}_{1.21\times10^{8}}$	$\frac{49}{41}{}_{4.5\times10^{8}}$	$\frac{117}{105}{}_{1.11\times10^{8}}$	$\frac{259}{241}{}_{2.5\times10^{8}}$	$\frac{134}{128}{}_{1.31\times10^{8}}$	$\frac{26}{27}{}_{2.6\times10^{8}}$	$\frac{81}{79}{}_{8.0\times10^{7}}$	$\frac{26}{19}{}_{2.2\times10^{8}}$	$\frac{83}{79}{}_{8.1\times10^{7}}$
Cell count following washing	$\frac{146}{139}{}_{1.42\times10^{7}}$	$\frac{64}{50}{}_{5.7\times10^{7}}$	$\frac{169}{196}{}_{1.82\times10^{8}}$	$\frac{37}{59}{}_{4.8\times10^{7}}$	$\frac{112}{112}{}_{1.12\times10^{8}}$	$\frac{59}{70}{}_{6.4\times10^{7}}$	$\frac{129}{146}{}_{1.38\times10^{8}}$	$\frac{72}{78}{}_{7.5\times10^{7}}$	$\frac{102}{90}{}_{9.6\times10^{7}}$	$\frac{50}{33}{}_{4.2\times10^{7}}$

X = No S-9 mix added
A = 0.5 S-9 mix added
TNTC = Too numerous to count

TABLE 15. Preincubation Test of Beef using Strain TA 98

	Beef									
	Saline Control		TPB		FB		GIB		EIB	
	X	A	X	A	X	A	X	A	X	A
Plate Incorporation Test (Revertants/plates)	359 217 285	94 89 120	63 75 72	23 25 46	61 67 68	24 28 29	7 9 14	12 7 14	18 17 10	32 23 27
Revertant Average	287	101	70	31	65	27	10	11	15	27
Initial Viable Cell Count	202 199 2.00×10^9		162 149 1.56×10^9		249 234 2.42×10^9		161 133 1.47×10^9		173 184 1.78×10^9	
Cell Count following 2 hr incubation with beef	52 56 5.4×10^8	26 36 3.1×10^8	83 82 8.2×10^8	20 22 2.1×10^8	47 24 3.6×10^8	29 24 2.6×10^8	60 63 6.2×10^8	157 160 1.58×10^8	51 56 5.4×10^8	20 30 2.5×10^8
Cell Count following washing	31 43 3.7×10^8	163 179 1.71×10^8	52 54 5.3×10^8	24 14 1.9×10^8	27 41 3.4×10^8	53 58 5.6×10^7	35 23 2.9×10^8	133 145 1.38×10^8	50 54 5.2×10^8	117 127 1.22×10^8

X = No S-9 mix added
A = 0.5 S-9 mix added

TABLE 16

TABLE 16. Preincubation Test of Beef using Strain TA 100

	Saline Control		Beef							
			TPB		FB		GIB		EIB	
	X	A	X	A	X	A	X	A	X	A
Plate Incorporation Test (Revertants/plates)	443 522 296	362 330 356	285 231 253	437 409 311	376 270 231	363 472 313	590 566 464	310 293 300	446 426 337	339 315 354
Revertant Average	420	349	256	386	292	383	540	301	403	336
Initial Viable Cell Count	$\frac{63}{\frac{34}{4.8\times10^{5}}}$		$\frac{64}{\frac{57}{6.0\times10^{5}}}$		$\frac{72}{\frac{80}{7.6\times10^{5}}}$		$\frac{89}{\frac{95}{9.2\times10^{4}}}$		$\frac{106}{\frac{94}{1.00\times10^{6}}}$	
Cell Count following 2 hr incubation with beef	$\frac{27}{\frac{13}{2.0\times10^{5}}}$	$\frac{67}{\frac{57}{6.2\times10^{5}}}$	$\frac{62}{\frac{57}{5.8\times10^{5}}}$	$\frac{60}{\frac{68}{6.4\times10^{5}}}$	$\frac{118}{\frac{80}{9.9\times10^{5}}}$	$\frac{107}{\frac{187}{1.47\times10^{5}}}$	$\frac{13}{\frac{15}{1.4\times10^{6}}}$	$\frac{31}{\frac{41}{3.6\times10^{5}}}$	$\frac{63}{\frac{69}{6.6\times10^{7}}}$	$\frac{39}{\frac{17}{2.8\times10^{5}}}$
Cell Count following washing	$\frac{32}{\frac{27}{3.0\times10^{4}}}$	$\frac{89}{\frac{133}{1.11\times10^{5}}}$	$\frac{67}{\frac{59}{6.3\times10^{5}}}$	$\frac{22}{\frac{6}{1.4\times10^{5}}}$	$\frac{39}{\frac{73}{5.6\times10^{5}}}$	$\frac{51}{\frac{47}{4.9\times10^{5}}}$	$\frac{31}{\frac{30}{3.0\times10^{6}}}$	$\frac{70}{\frac{59}{6.4\times10^{5}}}$	$\frac{55}{\frac{38}{4.6\times10^{5}}}$	$\frac{52}{\frac{46}{4.9\times10^{5}}}$

X = No S-9 mix added
A = 0.5 S-9 mix added

TABLE 17. Plate Incorporation Test of Beef Using Top Agar with 0.43 mM Histidine + 0.5 mM Biotin

Test Material	Amount of Test Material Added	S-9 Mix Added	Revertant Count Per Plate (Avg.) Strain No. 98	100	1535	1537	1538
None	N/A	-	20,44,28 (31)	257,352,290 (300)	27,50,51 (43)	20,11,11 (14)	10,15,11 (12)
		+	27,32,31 (30)	153,129,108 (130)	22,14,9 (15)	50,62,61 (58)	26,31,18 (25)
TPB	0.2 ml	-	0,5,1 (2)	188,244,10 (147)	3,9,12 (8)	0,0,0	0,0,0
		+	0,18,9 (9)	185,269,182 (212)	0,0,0	0,0,0	0,0,0
FB	0.2 ml	-	4,9,1 (5)	213,201,112 (175)	8,11,12 (10)	0,0,0	0,0,0
		+	0,0,0	97,3,46 (49)	0,16,17 (11)	9,5,19 (11)	0,0,0
GIB	0.2 ml	-	*350,365,400 (372)	*100,500,200 (267)	*450,365,328 (381)	*415,385,440 (413)	*303,448,320 (357)
		+	*300,300,300 (300)	*400,400,425 (408)	*350,350,350 (350)	*200,200,200 (200)	*400,400,400 (400)
EIB	0.2 ml	-	0,0,3 (1)	175,173,169 (172)	25,7,6 (13)	0,0,2 (1)	0,0,0
		+	0,0,0	100,65,70 (78)	0,0,0	0,0,0	0,0,0

* Estimated values

TABLE 18. Plate Incorporation Test of Beef Using Top Agar with 0.43 mM Histidine + 0.5 mM Biotin

Strain Control Strains	Histidine Requirement	Ampicillin Resistance	UV	Sensitivity to Crystal Violet	Sensitivity to Desoxycholate	Spontaneous Revertants
1535	+	NT	+	13mm	16mm	26
1537	+	-	+	16mm	31mm	47
1538	+	NT	+	No zone	30mm	17
98	+	+	+	15mm	15mm	29
100	+	+	+	15mm	19mm	151
WT	-	NT	-	No zone	No zone	N/A

Positive Controls

Compound	Amount of Compound Added	S-9 mix Added	Strains 98	100	1535	1537	1538
AF	2 µg/0.1 ml	+	17,208	160,248			92,94
MNNG	2 µg/0.1 ml	-		205,194			
MNNG	20 µg/0.1 ml	-			980,906		
DMBA	20 µg/0.1 ml	+	56,47	416,380		60,57	25,28
BP	2 µg/0.1 ml	+	41,38	276,261		96,28	63,46

Compound	S-9 mix Added	Strain	True Revertants/# tested 98	100	1535	1537	1538
TPB	-		2/3	18/18	3/3		
	+		4/4	18/18			
FB	-		3/3	18/18	3/3		
	+			16/18	2/3	2/3	
GIB	-		14/18	18/18	18/18	15/18	12/18
	+		4/18	14/18	8/18	5/12	8/18
LIB	-		1/2	18/18	5/5	2/2	
	+			18/18			

TABLE 19. Plate Incorporation Test Using Top Agar Containing 0.43 mM Histidine and No Biotin

Test Material	Amount of Test Material Added	S-9 Mix Added	Revertant Count Per Plate (Avg.) Strains 98	100	1535	1537	1538
None	N/A	-	25,9,19 (18)	140,231,298 (223)	39,38,33 (37)	13,10,8 (10)	4,7,15 (9)
None	N/A	+	53,36,44 (44)	221,239,229 (230)	28,30,34 (31)	16,20,13 (16)	25,40,20 (28)
TPB	0.2 ml	-	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0,0,0	0,0,0	0,0,0	14,0,1 (5)	0,0,0
FB	0.2 ml	-	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
GIB	0.2 ml	-	0,0,0	0,0,0	0,33,0 (11)	0,0,0	0,0,0
		+	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
EIB	0.2 ml	-	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0

TABLE 20. Plate Incorporation Test Using Top Agar Containing 0.5 mM Biotin and No Added Histidine

Test Material	Amount of Test Material Added	S-9 Mix Added	Revertant Count Per Plate (Avg.) Strains 98	100	1535	1537	1538
None	N/A	-	25,9,19 (18)	140,231,296 (223)	39,38,33 (37)	13,10,8 (10)	4,7,15 (9)
None	N/A	+	53,36,44 (44)	221,239,229 (230)	28,30,34 (31)	16,20,13 (16)	25,40,20 (28)
TPB	0.2 ml	-	79,108,72 (86)	233,276,236 (248)	26,29,24 (26)	16,7,15 (13)	6,23,13 (14)
		+	38,41,33 (37)	164,213,171 (183)	23,24,12 (20)	4,7,7 (6)	43,41,29 (38)
FB	0.2 ml	-	29,46,51 (42)	229,207,187 (208)	39,46,45 (43)	3,10,6 (6)	3,6,3 (4)
		+	41,18,35 (31)	206,129,158 (164)	22,12,12 (15)	3,3,4 (3)	20,29,35 (28)
GIB	0.2 ml	-	47,62,69 (59)	295,264,218 (259)	36,53,47 (45)	9,16,11 (12)	7,8,13 (9)
		+	35,34,36 (35)	151,168,298 (206)	15,18,19 (17)	64,39,55 (53)	35,31,32 (33)
EIB	0.2 ml	-	14,22,29 (22)	190,177,348 (238)	26,28,30 (28)	15,19,17 (17)	7,10,1 (6)
		+	38,30,45 (38)	180,257,256 (231)	18,16,17 (17)	11,11,6 (9)	22,26,30 (26)

TABLE 21. Plate Incorporation Test Using Top Agar Containing 0.43 mM Histidine + 0.5 mM Biotin

Test Material	Amount of Test Material Added	S-9 Mix Added	Revertant Count Per Plate (Avg.) Strains 98	100	1535	1537	1538
None	N/A	-	25,9,19 (18)	140,231,298 (223)	39,38,33 (37)	13,10,8 (10)	4,7,15 (9)
None	N/A	+	53,36,44 (44)	221,239,229 (230)	28,30,34 (31)	16,20,13 (16)	25,40,20 (28)
TPB	0.2 ml	-	6,39,16 (20)	327,390,275 (331)	51,47,51 (50)	28,18,25 (24)	0,7,5 (4)
		+	15,30,28 (24)	165,97,120 (127)	19,27,30 (25)	25,21,33 (26)	4,5,20 (10)
FB	0.2 ml	-	13,9,5 (9)	165,157,134 (152)	14,7,29 (17)	0,4,12 (5)	0,0,0
		+	0,5,5 (3)	97,75,62 (78)	5,16,26 (16)	5,0,0 (2)	8,0,0 (3)
GIB	0.2 ml	-	7,9,2 (6)	85,125,60 (90)	28,30,0 (19)	5,3,0 (3)	0,0,0
		+	22,1,30 (18)	85,139,157 (127)	31,18,3 (17)	10,0,0 (3)	12,8,5 (8)
EIB	0.2 ml	-	10,12,0 (7)	65,47,129 (80)	12,22,12 (15)	3,6,0 (3)	0,0,0
		+	0,6,14 (7)	97,65,67 (76)	5,10,2 (6)	9,12,8 (10)	4,0,0 (1)

TABLE 22. Plate Incorporation Test *Using Top Agar Without* Histidine or Biotin

Test Material	Amount of Test Material Added	S-9 Mix Added	Revertant Count Per Plate (Avg.) Strains 98	100	1535	1537	1538
None	N/A	-	25,9,19 (18)	140,231,298 (223)	39,38,33 (37)	13,10,8 (10)	4,7,15 (9)
None	N/A	+	53,36,44 (44)	221,239,229 (230)	28,30,34 (31)	16,20,13 (16)	25,40,20 (28)
TPB	0.2 ml	-	0,0,0	8,4,2 (5)	0,0,0	44,19,15 (26)	0,0,0
		+	0,0,0	0,0,0	0,0,0	46,50,50 (49)	0,0,0
FB	0.2 ml	-	0,0,0	0,0,0	0,1,0	0,11,1 (4)	0,1,0
		+	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
GIB	0.2 ml	-	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
EIB	0.2 ml	-	0,0,0	0,0,0	0,0,0	0,0,6 (2)	0,0,0
		+	0,0,0	0,1,0	0,0,0	0,0,0	0,0,0

TABLE 23. Testing the Effect of Varying the Histidine and Biotin Concentrations in the Top Agar when Testing Frozen, Thermal, Electron-Irradiated and Gamma-Irradiated Beef

Strain Control

Strain No.	Histidine Requirement	Ampicillin Resistance	Sensitivity to UV	Sensitivity to Crystal Violet	Sensitivity to Desoxycholate	Spontaneous Revertants
1535	+	NT	+	12 mm	15 mm	77
1537	+	-	+	15 mm	16 mm	12
1538	+	NT	+	19 mm	20 mm	4
98	+	+	+	10 mm	15 mm	44
100	+	+	+	14 mm	15 mm	473
WT	No growth	NT	-	no zone	no zone	N/A

Positive Controls

Compound	Amount of Compound Added	S-9 Mix Added	Revertants Per Plate (Avg.) Strains 98	100	1535	1537	1538
AF	2 µg/0.1 ml	+	65,162 (114)	160,195 (178)	-	-	42,42 (42)
MNNG	2 µg/0.1 ml	-	-	245,150 (198)	-	-	-
MNNG	20 µg/0.1 ml	-	-	-	1594,1595 (1594)	-	-
DMBA	20 µg/0.1 ml	+	27,73 (50)	605,442 (524)	-	39,36 (38)	13,23 (18)
BP	2 µg/0.1 ml	+	91,70 (80)	474,458 (466)	-	48,62 (55)	67,47 (57)

TABLE 24. Preincubation Test of Beef using Strain TA 98

	Saline control		Beef TPb		FB		GIB		LIB	
	X	A	X	A	X	A	X	A	X	A
Plate Incorporation Test (Revertants/plates)	200 * *	*,*,*	*,*,*	*,*,*	*,*,*	*,*,*	*,*,*	*,*,*	*,*,*	*,*,*
Revertant Average										
Initial Viable Cell Count	2.28×10^9		1.74×10^9		1.28×10^9		1.56×10^9		1.69×10^9	
Cell count following 2 hr incubation with beef	5.1×10^8	2.98×10^8	8.0×10^8	2.37×10^8	3.4×10^8	2.7×10^8	5.8×10^8	2.87×10^9	5.8×10^8	2.13×10^9
Cell Count following washing	5.1×10^7	2.07×10^8	4.3×10^8	3.1×10^8	3.5×10^8	1.58×10^9	4.9×10^8	1.66×10^8	5.6×10^8	1.88×10^8

X = No S-9 mix added
A = 0.5 S-9 mix added
* = Abnormal lawn

TABLE 25. Preincubation Test of Beef using Strain TA 100

	Saline Control		Beef							
			TPB		FB		GIB		EIB	
	X	A	X	A	X	A	X	A	X	A
Plate Incorporation Test (Revertant/plates	439 523 439	616 724 634	529 372 346	320 352 315	463 385 429	670 605 608	420 634 570	582 304 682	589 628 627	627 604 550
Revertant Average	467	658	416	329	426	628	541	523	615	594
Initial Viable Cell Count	3.9×10^6		4.5×10^6		1.94×10^6		4.5×10^6		4.9×10^6	
Cell Count following 2 hr incubation with beef	6.5×10^5	8.6×10^5	5.4×10^6	1.1×10^6	5.4×10^6	8.7×10^6	7.7×10^6	1.53×10^7	5.9×10^6	1.17×10^6
Cell Count following washing	1.92×10^5	8.9×10^5	1.82×10^6	5.8×10^5	8.1×10^5	7.9×10^5	2.19×10^6	1.74×10^6	8.7×10^8	1.36×10^6

X = No S-9 mix added
A = 0.5 S-9 mix added

TABLE 26. Preincubation Test of Beef using Strain TA 1535

	Beef									
	Saline Control		TPB		FB		GIB		EIB	
	X	A	X	A	X	A	X	A	X	A
Plate Incorporation Test (Revertant/plates)	0	0	0	31	0	25	0	14	1	27
	0	0	0	39	0	17	0	11	1	34
	0	0	0	27	0	42	1	12	0	39
Revertant Average				32		27		12	1	33
Initial Viable Cell Count	6.7×10^6		1.48×10^6		1.52×10^6		5.5×10^6		8.0×10^6	
Cell Count following 2 hr incubation with beef	6.0×10^5	1.07×10^6	1.32×10^6	9.1×10^5	5.2×10^6	7.5×10^5	1.75×10^6	5.4×10^5	1.22×10^7	9.3×10^5
Cell Count following washing	4.0×10^7	1.37×10^7	7.8×10^7	5.9×10^6	6.1×10^7	4.1×10^6	7.8×10^7	7.1×10^6	6.6×10^7	1.17×10^8

X = No S-9 mix added
A = 0.5 S-9 mix added

TABLE 27. Preincubation Test of Beef using Strain TA 1537

	Saline Control		Beef							
			TPB		FB		GIB		EIB	
	X	A	X	A	X	A	X	A	X	A
Plate Incorporation Test (Revertant/plates)	2	20	0	5	NL	7	NL	2	2	2
	5	20	0	4	NL	0	NL	1	2	2
	1	12	0	2	NL	NL	NL	4	0	NL
Revertant Average	3	17		4		2		2	1	1
Initial Viable Cell Count	4.9×10^8		1.07×10^9		7.2×10^8		6.0×10^8		5.8×10^8	
Cell Count following 2 hr incubation with beef	7.0×10^7	1.25×10^8	2.05×10^8	1.32×10^8	1.51×10^8	1.17×10^8	2.01×10^8	1.14×10^8	4.4×10^8	1.75×10^8
Cell Count following washing	6.9×10^7	7.7×10^7	1.95×10^8	1.04×10^8	5.0×10^7	6.9×10^7	1.49×10^8	5.6×10^7	1.15×10^8	6.4×10^7

X - NO S-9 mix added
A - 0.5 S-9 mix added
NL = No lawn

TABLE 28. Preincubation Test of Beef using Strain TA 1538

	Beef									
	Saline Control		TPB		FB		GIb		EIB	
	X	A	X	A	X	A	X	A	X	A
Plate Incorporation Test (Revertants/plate)	IL	7	IL	1	IL	2	IL	5	IL	4
	IL	8	IL	2	IL	3	IL	4	IL	6
	IL	4	IL	4	IL	2	IL	2	IL	3
REvertant AVerage		6		2		2		4		4
Initial Viable Cell Count	6.2×10^8		4.7×10^8		3.7×10^8		6.2×10^8		5.5×10^8	
Cell Count following 2 hr incubation with beef	3.4×10^7	7.7×10^7	2.4×10^8	1.14×10^8	1.31×10^8	9.2×10^7	3.0×10^8	9.5×10^7	3.4×10^8	1.26×10^8
Cell Count following washing	2.9×10^7	6.6×10^7	3.7×10^8	1.38×10^8	1.07×10^8	9.8×10^7	2.19×10^8	8.3×10^7	3.7×10^7	1.20×10^8

X = No S-9 mix added
A = 0.5 S-9 mix added
IL = Incomplete lawn

Table 29. Plate Incorporation Test of Beef with Altered Levels of Histidine and Biotin in Top Agar

Test Material	Amount added to Plate	S-9 mix Added	Top Agar Additive	Strain	Revertant Count per Plate 98	100	1535	1537	1538
None	N.A	-	0.5mM Histidine 0.5mM Biotin		32,35 (34)	172,172 (172)	26,19 (23)	13,15 (14)	11,7 (9)
		+	0.5mM Histidine 0.5mM Biotin		39,51 (45)	193,158 (176)	10,7 (9)	10,17 (9)	31,30 (31)
TPB	0.2ml	-	0.43mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0.43mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		-	0.5mM Biotin		95,179,172 (149)	210,208,238 (219)	14,7,14 (12)	2,3,0 (2)	140,165,279 (195)
		+	0.5mM Biotin		83,65,73 (74)	418,1006,678 (701)	14,20,16 (17)	6,10,4 (7)	79,84,68 (77)
		-	0.43mM Histidine 0.5mM Biotin		43,28,32 (34)	203,167,149 (173)	30,66,38 (45)	0,0,0	0,7,0 (2)
		+	0.43mM Histidine 0.5mM Biotin		48,44,28 (40)	239,216,222 (226)	18,12,25 (15)	8,6,5 (3)	30,27,14 (24)
		-	0.5mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0.5mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
Glb	0.2ml	-	0.43mM Histidine		24,29,0 (18)	388,318,0 (235)	0,0,0	0,0,0	7,0,0 (2)
		+	0.43mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		-	0.5mM Biotin		23,22,15 (20)	377,332,197 (302)	18,22,14 (18)	0,0,0	0,0,0
		+	0.5mM Biotin		44,50,59 (51)	565,291,870 (575)	21,24,34 (26)	7,4,4 (5)	23,24,27 (25)
		-	0.43mM Histidine 0.5mM Biotin		23,46,25 (31)	0,0,0	0,0,0	2,0,4 (2)	11,15,13 (14)
		+	0.43mM Histidine 0.5mM Biotin		47,33,37 (39)	302,232,302 (279)	27,22,26 (25)	4,5,3 (4)	30,27,30 (29)
		-	0.5mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0.5mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0

TABLE 30 Controls for Test 10
Controls fo Test 10

Strain Control

Strain No.	Histidine Requirement	Ampicillin Resistance	Sensitivity to UV	Sensitivity to Crystal Violet	Sensitivity to Desoxycholate	Spontaneous Revertants
1535	+	NT	+	16 mm	14 mm	18,19 (19)
1537	+	-	+	14 mm	16 mm	22,29 (26)
1538	+	NT	+	15 mm	16 mm	13,13 (13)
98	+	+	+	16 mm	13 mm	29,29 (29)
100	+	+	+	15 mm	14 mm	167,151 (159)
WT	-	+	-	no zone	no zone	N/A

Positive Controls

Compound	Amount of Compound Added	S-9 Mix Added	Revertants Per Plate (Avg.) Strains 98	100	1535	1537	1538
AF	2 µg/0.1 ml	+	168,164 (166)	203,144 (174)	-	-	156,188 (172)
MNNG	2 µg/0.1 ml	-	-	756,704 (730)	-	-	-
MNNG	20 µg/0.1 ml	-	-	-	2290,2226 (2258)	-	-
DMBA	20 µg/0.1 ml	+	158,101 (130)	630,621 (626)	-	48,71 (60)	45,42 (44)
BP	2 µg/0.1 ml	+	292,401 (346)	88,107 (98)	-	62,92 (77)	106,70 (88)

Table 32 Plate Incorporated Test of Beef with Altered Levels of Histidine and Biotin in Top Agar

Test Material	Amount added to Plate	S-9 mix Added	Top Agar Additive	Strain	Revertant Count per Plate 98	100	1535	1537	1538
None	N/A	-	0.5mM Histidine 0.5mM Biotin		24,31 (28)	93,153 (123)	13,17 (15)	6,3 (5)	7,8 (8)
		+	0.5mM Histidine 0.5mM Biotin		37,30 (34)	92,89 (91)	7,22 (15)	14,11 (13)	16,15 (16)
FB	0.2ml	-	0.43mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0.43mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		-	0.5mM Biotin		26,20,LA	202,275,219 (232)	11,2,3 (5)	0,0,0	29,24,14 (22)
		+	0.5mM Biotin		47,32,45 (41)	*536,536,536 (536)	22,39,60 (40)	0,0,0	3,1,5 (5)
		-	0.43mM Histidine 0.5mM Biotin		46,30,47 (41)	405,320,407 (377)	57,57,63 (59)	8,4,3 (5)	13,15,16 (15)
		+	0.43mM Histidine 0.5mM Biotin		35,52,64 (50)	LA,LA,LA	50,43,58 (50)	4,6,2 (4)	15,9,14 (13)
		-	0.5mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0.5mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
EIb	0.2ml	-	0.43mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0.43mM Histidine		0,0,0	0,0,0	66,52,12 (43)	0,0,0	0,0,0
		-	0.5mM Biotin		49,38,29 (39)	385,214,327 (309)	44,36,31 (37)	4,7,5 (5)	4,10,6 (7)
		+	0.5mM Biotin		35,66,48 (50)	*300,300,300 (300)	31,22,53 (35)	4,9,3 (5)	27,20,25 (24)
		-	0.43mM Histidine 0.5mM Biotin		25,28,16 (23)	278,275,333 (295)	11,26,29 (22)	1,2,2 (2)	2,6,5 (4)
		+	0.43mM Histidine 0.5mM Biotin		21,39,38 (33)	349,292,400 (347)	39,28,30 (32)	3,4,7 (5)	12,12,14 (13)
		-	0.5mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0.5mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0

LA = Lab Accident plates uncountable

* = Estimated values

TABLE 32. Controls for Test 11

Strain Control Strain No.	Histidine Requirement	Ampicillin Resistance	Sensitivity to UV	Sensitivity to Crystal Violet	Sensitivity to Desoxycholate	Spontaneous Revertants
1535	+	NT	+	15 mm	13 mm	16,26 (21)
1537	+	-	+	15 mm	16 mm	34,32 (33)
1538	+	NT	+	16 mm	16 mm	10,6 (8)
98	+	+	+	14 mm	14 mm	33,32 (33)
100	+	+	+	14 mm	14 mm	153,154 (154)
WT	-	NT	-	-	-	N/A

Positive Controls Compound	Amount of Compound Added	S-9 Mix Added*	Revertants Per Plate (Avg.) Strains 98	100	1535	1537	1538
AF	2 µg/0.1 ml	+	135,160 (148)	77,80 (78)	-	-	150,121 (136)
MNNG	2 µg/0.1 ml	-	-	1889,1689 (1789)	-	-	-
MNNG	20 µg/0.1 ml	-	-	-	1701,1897 (1799)	-	-
DMBA	20 µg/0.1 ml	+	85,101 (93)	89,34 (62)	-	36,52 (44)	46,26 (36)
BP	2 µg/0.1 ml	+	389,346 (368)	2,2 (2)	-	39,25 (32)	106,102 (104)

* + = 0.5 ml S-9 Mix Added/Plate or positive response (as appropriate)
- = No S-9 Mix Added or negative response (as appropriate)

Table 31. Plate Incorporation Test of Beet with Altered Levels of Histidine and Biotin in Top Agar

Test Material	Amount added to Plate	S-9 Mix Added	Top Agar Additive	Strain	Revertant Count per Plate 98	100	1535	1537	1538
None	N.A.	-	0.5mM Histidine 0.5mM Biotin		22,15 (19)	144,136 (140)	8,11 (10)	7,2 (5)	3,2 (3)
		+	0.5mM Histidine 0.5mM Biotin		34,28 (31)	72,70 (71)	10,7 (9)	12,15 (14)	27,21 (24)
TPB	0.2ml	-	0.43mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0.43mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		-	0.5mM Biotin		29,30,12 (24)	382,376,373 (377)	30,25,22 (26)	0,1,0	1,0,0
		+	0.5mM Biotin		35,23,36 (31)	305,237,203 (248)	26,15,37 (26)	2,2,3 (2)	15,23,17 (18)
		-	0.43mM Histidine 0.5mM Biotin		9,7,0 (5)	340,305,339 (328)	21,26,33 (27)	0,0,2 (1)	0,0,0
		+	0.43mM Histidine 0.5mM Biotin		28,35,38 (34)	*,*,*	16,18,21 (18)	4,5,7 (5)	12,13,9 (11)
		-	0.5mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0.5mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
CIB	0.2ml	-	0.43mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0.43mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		-	0.5mM Biotin		14,22,21 (19)	353,413,339 (368)	20,30,28 (26)	0,0,0	0,0,0
		+	0.5mM Biotin		40,25,37 (34)	314,539,555 (469)	19,*,0 (6)	0,0,0	5,1,5 (4)
		-	0.43mM Histidine 0.5mM Biotin		5,0,26 (14)	429,396,386 (404)	43,27,35 (35)	0,0,0	*,0,0
		+	0.43mM Histidine 0.5mM Biotin		34,13,21 (23)	287,556,530 (458)	*,14,18 (11)	0,0,0	0,0,0
		-	0.5mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0.5mM Histidine		0,0,0	0,0,0	0,0,0	0,0,0	0,0,0

* Lawn & Revertants indistinguishable

TABLE 34. Controls for Test 12; Plate Incorporation Test of Beef with Altered Levels of Histidine and Biotin in the Top Agar

Strain Control

Strain No.	Histidine Requirement	Ampicillin Resistance	Sensitivity to UV	Sensitivity to Crystal Violet	Sensitivity to Desoxycholate	Spontaneous Revertants
1535	+	NT	+	15 mm	15 mm	10,13 (12)
1537	+	-	+	15 mm	16 mm	14,16 (15)
1538	+	NT	+	16 mm	16 mm	6,10 (8)
98	+	+	+	11 mm	14 mm	35,35 (35)
100	+	+	+	15 mm	13 mm	148,154 (151)
WT	-	NT	-	-	-	N/A

Positive Controls

Compound	Amount of Compound Added*	S-9 Mix Added	Revertants Per Plate (Avg.) Strains 98	100	1535	1537	1538
AF	2 µg/0.1 ml	+	69,77 (73)	7,13 (10)	-	-	48,39 (44)
MNNG	2 µg/0.1 ml	-	-	236,232 (234)	-	-	-
MNNG	20 µg/0.1 ml	-	-	-	920,511 (716)	-	-
DMBA	20 µg/0.1 ml	+	68,54 (61)	105,170 (138)	-	30,30 (30)	30,29 (30)
BP	2 µg/0.1 ml	+	130,220 (175)	21,18 (20)	-	7	44,41 (42)

* + = 0.5 ml S-9 Mix Added/Plate
  - = No S-9 Mix Added

TABLE 35. Plate Incorporation Test Using Top Agar Containing 0.5 mM Histidine

Test Material	Amount of Test Material Added	S-9 Mix Added	Revertant Count Per Plate (Avg.) Strains 98	100	1535	1537	1538
None	N/A	-	32,27,27 (29)	121,96,94 (104)	18,12,9 (13)	12,6,9 (9)	7,1,2 (3)
None	N/A	+	33,27,29 (30)	133,157,128 (139)	5,9,6 (7)	10,15,5 (10)	13,15,10 (13)
FB	0.2 ml	-	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
TPB	0.2 ml	-	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0,4,0	4,0,0	0,0,2	0,0,0	0,0,0
GIB	0.2 ml	-	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
EIB	0.2 ml	-	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0

TABLE 36. Plate Incorporation Test Using Top Agar Containing 0.43 mM Histidine + 0.5 mM Biotin

Test Material	Amount of Test Material Added	S-9 Mix Added	Revertant Count Per Plate (Avg.) Strains 98	100	1535	1537	1538
None	N/A	-	32,27,27 (29)	121,96,94 (104)	18,12,9 (13)	12,6,9 (9)	7,1,2 (3)
None	N/A	+	33,27,29 (30)	133,157,128 (139)	5,9,6 (7)	10,15,5 (10)	13,15,10 (13)
TPB	0.2 ml	-	92,36,54 (61)	387,263,282 (311)	36,59,60 (52)	10,6,5 (7)	10,4,3 (6)
		+	47,36,38 (40)	277,299,276 (284)	22,28,25 (25)	1,4,5 (3)	3,3,3 (3)
FB	0.2 ml	-	58,48,54 (53)	504,358,402 (421)	46,30,44 (40)	5,4,3 (4)	9,4,0 (4)
		+	20,41,27 (29)	256,224,301 (260)	28,41,42 (37)	2,3,2 (2)	5,5,4 (5)
GIB	0.2 ml	-	44,31,31 (35)	374,360,287 (340)	26,17,29 (24)	3,3,7 (4)	3,8,4 (5)
		+	23,14,43 (27)	167,181,176 (175)	33,35,26 (31)	3,2,1 (2)	4,2,5 (4)
EIB	0.2 ml	-	35,58,38 (44)	232,209,186 (209)	23,36,34 (31)	3,3,4 (3)	2,5,2 (3)
		+	42,18,42 (34)	284,236,395 (305)	26,26,16 (23)	6,8,5 (6)	2,0,6 (3)

TABLE 37: Plate Incorporation Test Using Top Agar Containing 0.5 mM Biotin

Test Material	Amount of Test Material Added	S-9 Mix Added	Revertant Count Per Plate (Avg.) Strains 98	100	1535	1537	1538
None	N.A	-	32,27,27 (29)	121,96,94 (104)	18,2,9 (10)	12,6,9 (9)	7,1,2 (3)
None	N.A	+	33,27,29 (30)	133,157,128 (139)	5,9,6 (7)	10,15,5 (10)	13,15,10 (13)
IPB	0.2 ml	-	39,41,56 (45)	311,434,284 (343)	14,24,15 (18)	4,9,2 (5)	7,4,12 (8)
		+	38,39,48 (42)	333,245,232 (270)	36,29,51 (39)	6,3,5 (5)	6,11,6 (8)
PB	0.2 ml	-	46,41,42 (43)	362,426,215 (334)	41,36,32 (36)	4,3,6 (5)	10,12,12 (11)
		+	41,46,33 (40)	245,392,322 (320)	29,25,36 (30)	6,4,3 (4)	10,15,14 (13)
GIB	0.2 ml	-	61,34,30 (42)	160,90,41 (97)	8,36,16 (20)	3,4,2 (3)	5,6,4 (5)
		+	50,32,45 (42)	170,179,182 (177)	25,8,26 (20)	3,6,4 (4)	15,27,19 (20)
EIB	0.2 ml	-	65,37,45 (49)	190,178,190 (186)	28,30,19 (26)	3,8,4 (5)	11,4,4 (6)
		+	44,32,32 (36)	176,157,186 (173)	24,14,23 (20)	8,1,4 (4)	26,16,16 (19)

TABLE 38. Plate Incorporation Test Using Top Agar Containing 0.43 mM Histidine

Test Material	Amount of Test Material Added	S-9 Mix Added	Revertant Count Per Plate (Avg.) Strains 98	100	1535	1537	1538
None	N/A	-	32,27,27 (29)	121,96,94 (104)	18,2,9 (10)	12,6,9 (9)	7,1,2 (3)
None	N/A	+	33,27,29 (30)	133,157,128 (139)	5,9,6 (7)	10,15,5 (10)	13,15,10 (13)
TPB	0.2 ml	-	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0,0,0	0,0,0	0,0,0	0,0,0	0,0,1
FB	0.2 ml	-	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
GIB	0.2 ml	-	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
EIB	0.2 ml	-	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
		+	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0

Table 39. Controls for Plate Incorporation Test of Beef with Altered Levels of Histidine and Biotin in Top Agar

STRAIN CONTROL

Strain No.	Histidine Requirement	Ampicillin Resistance	Sensitivity to Crystal Violet UV	Crystal Violet	Desoxycholate	Spontaneous Revertants (Avg)
1535	+	NT	+	14mm	14mm	10,12 (11)
1537	+	-	+	15mm	20mm	11,11 (11)
1538	+	NT	+	16mm	22mm	11, 8 (10)
98	+	+	+	14mm	12mm	33,38 (36)
100	+	+	+	16mm	19mm	94,104 (99)
WT	-	NT	-	-	-	N/A

POSITIVE CONTROLS

Compound	Amount of Compound Added	S-9 Mix Added	Revertants per Plate (Avg) Strains 98	100	1535	1537	1538
AF	2µg/0.1ml	+	385,286,387 (353)	231,248,188 (222)			361,290,288 (313)
BP	2µg/0.1ml	+	149,161,133 (148)	293,267,281 (280)		47,71,59 (59)	79,94,104 (92)
MNNG	2µg/0.1ml	-		567,476,488 (510)			
MNNG	20µg/0.1ml	-			>1000,>1000,>1000 (>1000)		
DMBA	20µg/0.1ml	+	86,87,86 (86)	284,281,266 (277)		59,58,63 (60)	38,32,30 (33)

TABLE 40. Verification of Revertants Produced in Test 13

Test Material	Additive	S-9 Mix Added	Strain: No.True Revertants/No.Apparent Revertants Tested 98	100	1535	1537	1538
FB	0.5 mM Biotin	-	8/8	22/23	4/4	4/4	8/8
		+	8/8	15/30	2/2	1/2	0/1
FB	0.43 mM Histidine	-	1/4	--	--	--	--
	0.5 mM Biotin	+	1/8	20/26	--	5/7	3/4
TPB	0.5 mM Biotin	-	2/18	6/18	3/4	1/5	0/8
		+	0/23	6/28	--	3/14	3/8
TPB	0.5 mM Histidine	-	--	--	0/1	--	--
		+	1/1	0/1	--	--	--
TPB	0.43 mM Histidine	-	2/10	5/32	4/7	2/7	0/6
	0.5 mM Biotin	+	1/8	3/22	4/5	1/3	0/3
GIB	0.5 mM Biotin	-	8/8	18/20	4/4	4/4	5/8
		+	6/8	5/20	3/4	0/4	2/4
GIB	0.43 mM Histidine	-	8/8	24/34	--	--	--
	0.5 mM Biotin	+	5/6	6/6	1/3	0/2	2/4
EIB	0.5 mM Biotin	-	3/10	11/18	5/5	2/5	3/7
		+	3/7	4/17	2/4	2/4	3/4
EIB	0.43 mM Histidine	-	3/9	10/16	4/5	2/2	2/3
	0.5 mM Biotin	+	7/9	9/29	1/5	2/5	1/3
TOTAL			67/153	174/340	37/53	29/68	32/71
% TRUE REVERTANT			44	51	43	37	55

TABLE 41. Plate Incorporation Test of Beef Using Top Agar Containing 0.5 mM Histidine + 0.5 mM Biotin

Test Compound	Amount of Compound Added	S-9 Mix Added	Revertants Per Plate (Avg.) Strains 98	100	1535	1537	1538
None	N/A	-	39,41,31 (37)	159,136,136 (144)	14,10,9 (11)	3,5,9 (6)	11,3,8 (7)
None	N/A	+	33,35,27 (32)	130,179,149 (153)	10,10,14 (11)	6,7,7 (7)	17,15,15 (16)
TPB	0.2 ml	-	77,79,68 (75)	304,306,316 (309)	61,76,74 (70)	2,9,4 (5)	29,18,12 (20)
		+	59,60,44 (54)	300,254,344 (299)	49,33,22 (35)	3,7,10 (7)	13,18,11 (14)
FB	0.2 ml	-	82,67,77 (75)	309,293,292 (298)	32,33,42 (36)	1,3,7 (4)	22,10,17 (16)
		+	54,42,40 (45)	236,259,269 (255)	27,29,31 (29)	4,8,5 (6)	28,23,18 (23)
GIB	0.2 ml	-	49,46,50 (48)	298,344,231 (291)	29,49,51 (43)	3,5,10 (6)	24,24,23 (24)
		+	55,57,41 (51)	229,224,243 (232)	24,24,27 (25)	5,3,2 (3)	19,24,25 (23)
EIB	0.2 ml	-	57,44,37 (46)	342,218,295 (285)	44,38,38 (40)	5,5,3 (4)	19,11,18 (16)
		+	35,36,38 (36)	314,267,257 (279)	26,33,17 (25)	9,1,7 (6)	17,16,26 (20)

TABLE 42. Plate Incorporation Test of Water Extracts of Beef Using Top Agar with 0.5 mM Histidine + 0.5 mM Biotin

Test Compound	Amount of Compound Added	S-9 Mix Added	Revertants Per Plate (Avg.) Strains 98	100	1535	1537	1538
None	N/A	-	39,31,41 (37)	159,136,136 (144)	14,10,9 (11)	3,5,9 (6)	11,3,8 (7)
None	N/A	+	33,35,27 (32)	130,179,149 (153)	10,10,14 (11)	6,7,7 (7)	17,15,15 (16)
TPB	0.2 ml	-	81,84,77 (81)	295,265,222 (261)	48,38,35 (40)	8,13,5 (9)	34,31,25 (30)
		+	59,73,58 (63)	328,345,312 (328)	42,31,18 (30)	6,12,22 (13)	49,49,53 (50)
FB	0.2 ml	-	82,53,75 (70)	300,273,160 (244)	36,51,54 (47)	10,6,12 (9)	40,48,47 (45)
		+	72,103,70 (82)	324,287,259 (290)	30,38,40 (36)	19,9,12 (13)	45,40,64 (50)
GIB	0.2 ml	-	62,68,51 (60)	213,149,166 (176)	44,48,46 (46)	13,9,8 (10)	26,33,32 (30)
		+	71,67,74 (71)	270,215,197 (227)	29,29,24 (27)	12,17,20 (16)	33,39,35 (36)
EIB	0.2 ml	-	48,44,54 (49)	177,121,164 (154)	51,60,37 (49)	10,11,8 (10)	17,28,23 (23)
		+	74,63,59 (65)	217,175,88 (160)	20,24,30 (25)	14,4,11 (10)	47,41,59 (49)

TABLE 43. Control for Plate Incorporation Tests of Beef and Water Extracts of Beef

Strain Control

Strain No.	Histidine Requirement	Ampicillin Resistance	Sensitivity to UV	Sensitivity to Crystal Violet	Sensitivity to Desoxycholate	Spontaneous Revertants
1535	+	NT	+	15 mm	13 mm	29,21 (25)
1537	+	-	+	16 mm	20 mm	25,13 (19)
1538	+	NT	+	16 mm	24 mm	17,25 (21)
98	+	+	+	14 mm	13 mm	55,52 (54)
100	+	+	+	16 mm	21 mm	142,143 (143)
WT	-	NT	-	-	-	N/A

Positive Controls

Compound	Amount of Compound Added	S-9 Mix Added	Revertants Per Plate (Avg.) Strains 98	100	1535	1537	1538
AF	2 µg/0.1 ml	+	319,352,328 (333)	191,223,230 (215)	-	-	374,434,464 (424)
BP	2 µg/0.1 ml	+	199,173,195 (189)	301,361,323 (328)	-	66,59,60 (62)	104,139,122 (122)
MNNG	2 µg/0.1 ml	-	-	712,624,714 (683)	-	-	-
MNNG	20 µg/0.1 ml	-	-	-	>1000,>1000,>1000 (>1000)	-	-
DMBA	20 µg/0.1 ml	+	131,108,129 (123)	325,357,396 (359)	-	61,69,59 (63)	34,33,64 (44)

TABLE 44. Plate Incorporation Test of Water Extracts of Beef

Compound	Amount of Compound Added	S-9 Mix Added	Revertants per Plate (Avg.) Strains 98	100	1535	1537	1538
None	N/A	-	26,32,31 (30)	100,99,109 (103)	7,13,12 (11)	5,7,9 (7)	11,17,12 (13)
None	N/A	+	23,22,26 (24)	101,98,90 (96)	13,10,11 (11)	7,3,8 (6)	25,18,25 (23)
TPB	0.2 ml	-	76,78,50 (68)	188,177,234 (200)	76,54,40 (57)	8,9,8 (8)	22,24,20 (22)
		+	106,142,77 (108)	284,272,320 (292)	68,58,43 (56)	11,9,8 (9)	38,67,59 (55)
FB	0.2 ml	-	72,74,81 (76)	314,291,201 (269)	72,64,65 (67)	7,2,2 (4)	26,32,26 (28)
		+	64,50,70 (61)	144,220,215 (193)	38,42,63 (48)	14,4,15 (11)	32,29,29 (30)
GIB	0.2 ml	-	107,88,80 (92)	177,185,233 (198)	52,56,135 (81)	12,10,8 (10)	28,42,43 (38)
		+	55,57,92 (68)	198,211,254 (221)	46,53,49 (49)	4,8,5 (6)	34,24,51 (36)
EIB	0.2 ml	-	91,65,70 (75)	140,198, 159 (166)	55,52,70 (59)	6,9.16 (10)	28,31,23 (27)
		+	72,62,132 (89)	234,191,179 (201)	44,43,32 (40)	6,7,9 (7)	57,37,42 (45)

TABLE 45. Controls for Plate Incorporation Test of Water Extracts of Beef

Strain Control Strains	Histidine Requirement	Ampicillin Resistance	Sensitivity to UV	Sensitivity to Crystal Violet	Sensitivity to Desoxycholate	Spontaneous Revertants
1535	+	NT	+	14 mm	16 mm	19,6 (13)
1537	+	-	+	15 mm	20 mm	5,4 (5)
1538	+	NT	+	15 mm	20 mm	11,20 (15)
98	+	+	+	14 mm	14 mm	24,34 (29)
100	+	+	+	17 mm	20 mm	77,91 (84)
WT	-	NT	-	-	-	N/A

Compound	Amount of Compound Added	S-9 Mix Added	Revertants per Plate (Avg.) Strains 98	100	1535	1537	1538
AF	2 µg/0.1 ml	+	528,470,407 (468)	208,213,202 (208)	-	-	586,374,427 (396)
BP	2 µg/0.1 ml	+	211,194,203 (203)	332,351,342 (342)	-	83,64,58 (68)	111,123,111 (115)
MNNG	2 µg/0.1 ml	-	-	397,237,295 (310)	-	-	-
MNNG	20 µg/0.1 ml	-	-	-	>1000,>1000,>1000 (>1000)	-	-
DMBA	20 µg/0.1 ml	+	114,123,96 (111)	327,358,303 (329)	-	65,63,48 (59)	57,51,65 (58)

LIST OF ABBREVIATIONS

APPENDIX F

LIST OF ABBREVIATIONS

AF	2-aminofluorene
BP	benzo-α-pyrene
DMBA	dimethyl benzanthracene
DMSO	dimethylsulfoxide
MNNG	N-methyl-n'nitro-n-nitrosogurandine
EIB	electron-irradiated beef
GIB	gamma-irradiated beef
FB	frozen beef
TPB	thermally processed beef
S-9	liver microsomal preparation
LA	laboratory accident (sample lost)
NT	not tested
Mg	microgram
ug	microgram
*	as indicated by footnote
WT	wild type

FIGURES

APPENDIX F

LIST OF FIGURES

All figures are magnified approximately 2X

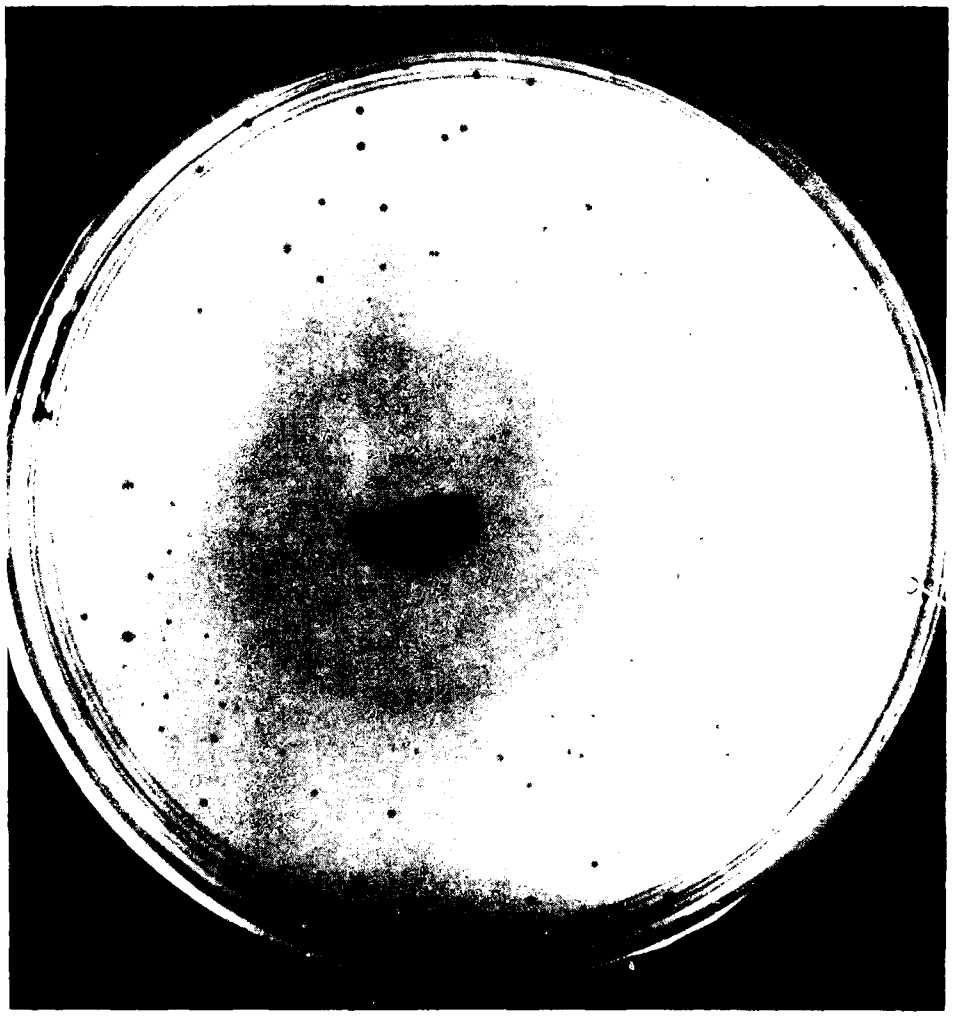
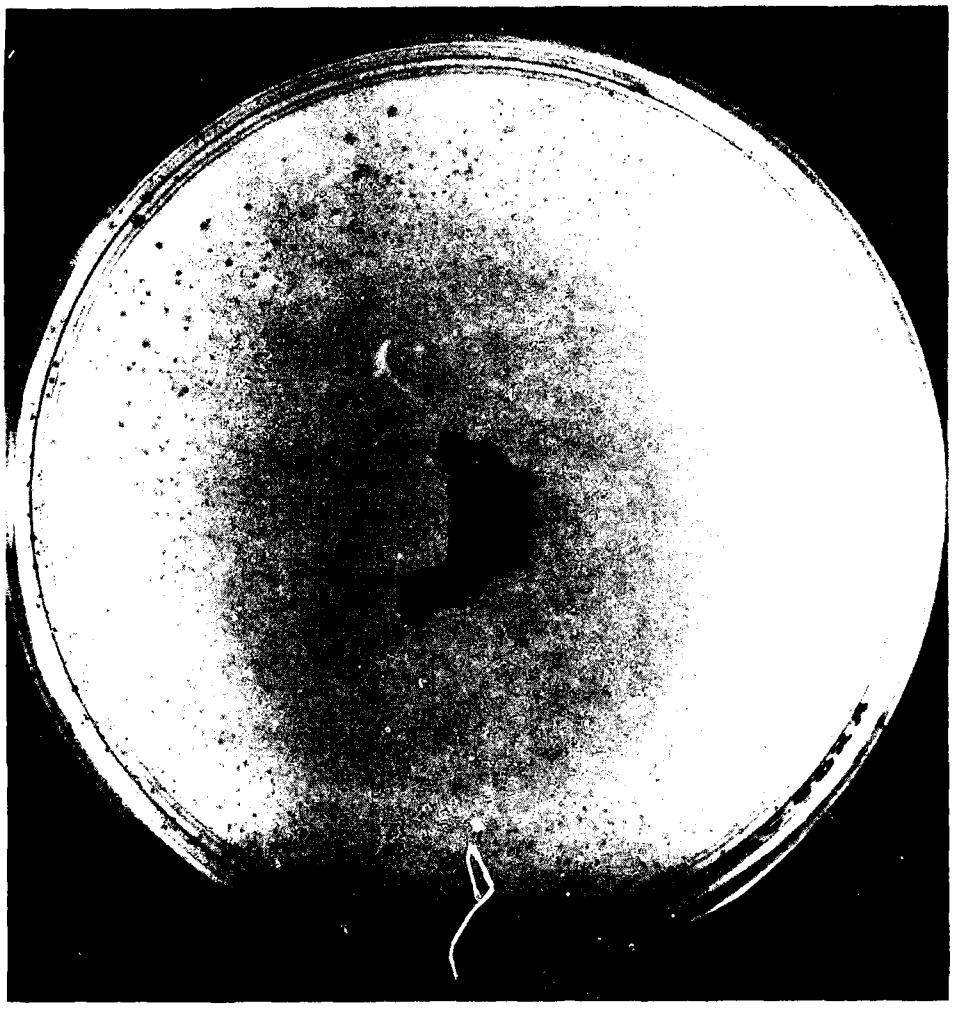


Figure 1. Spot Test of Gamma-Irradiated Beef, Strain TA100



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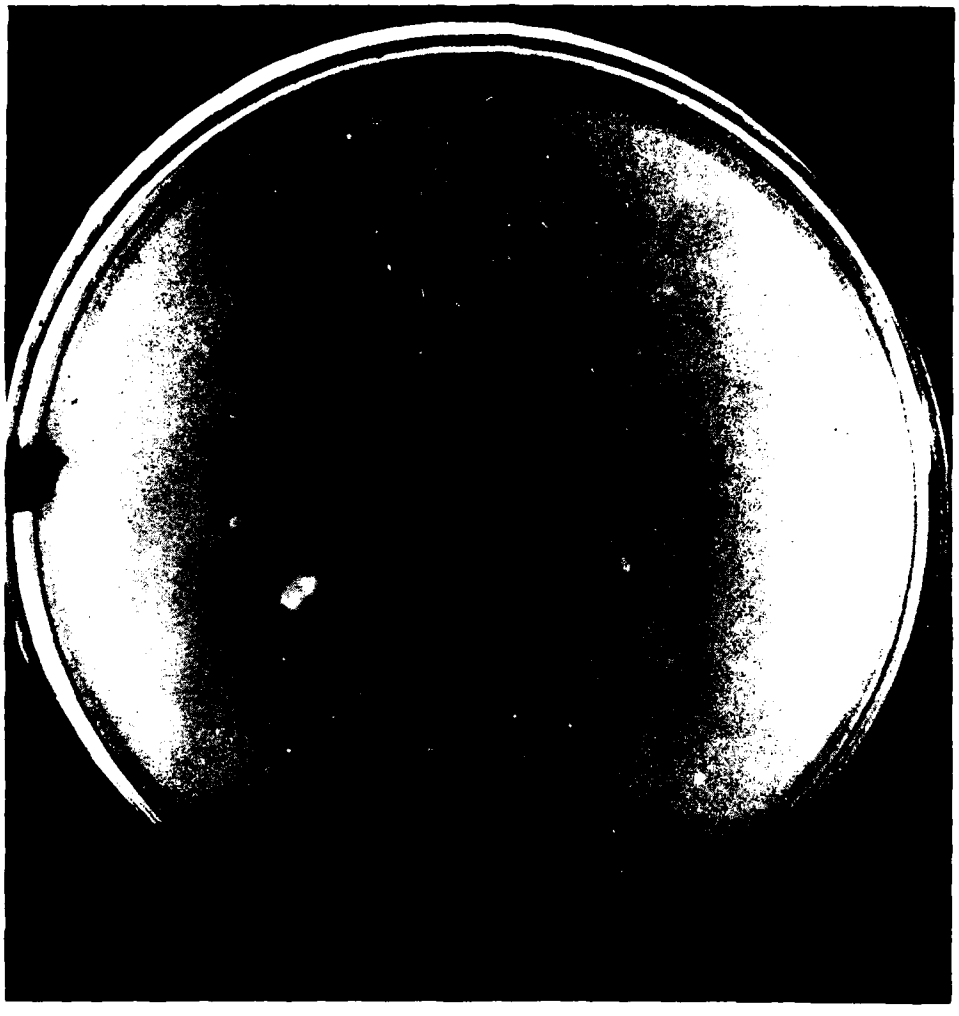

Figure 3. Spot Test of Gamma-Irradiated Beef, Wild Type, S. typhimurium.

Figure 4. Plate Incorporation Test of Thermally Processed Beef, Strain TA1535

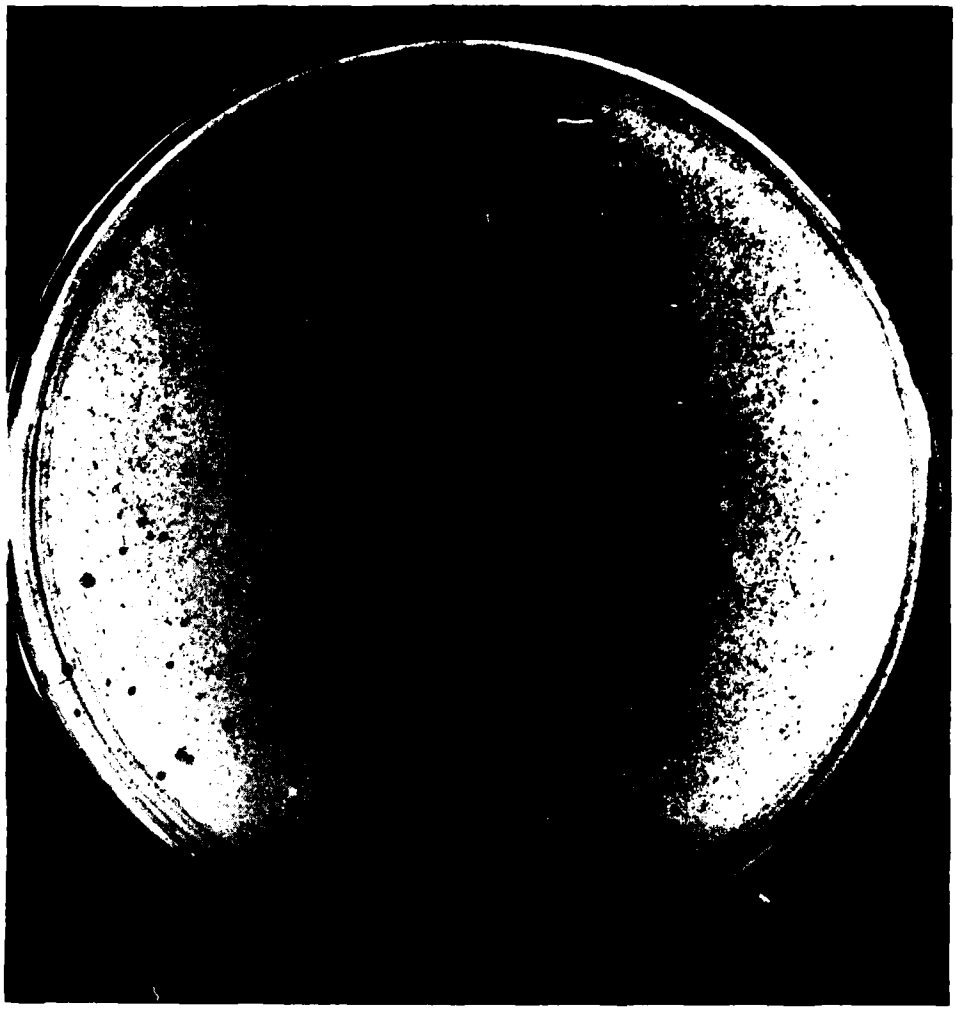

Figure 5. Plate Incorporation Test of Theramlly Processed Beef With 2 µg BP, Strain TA1538

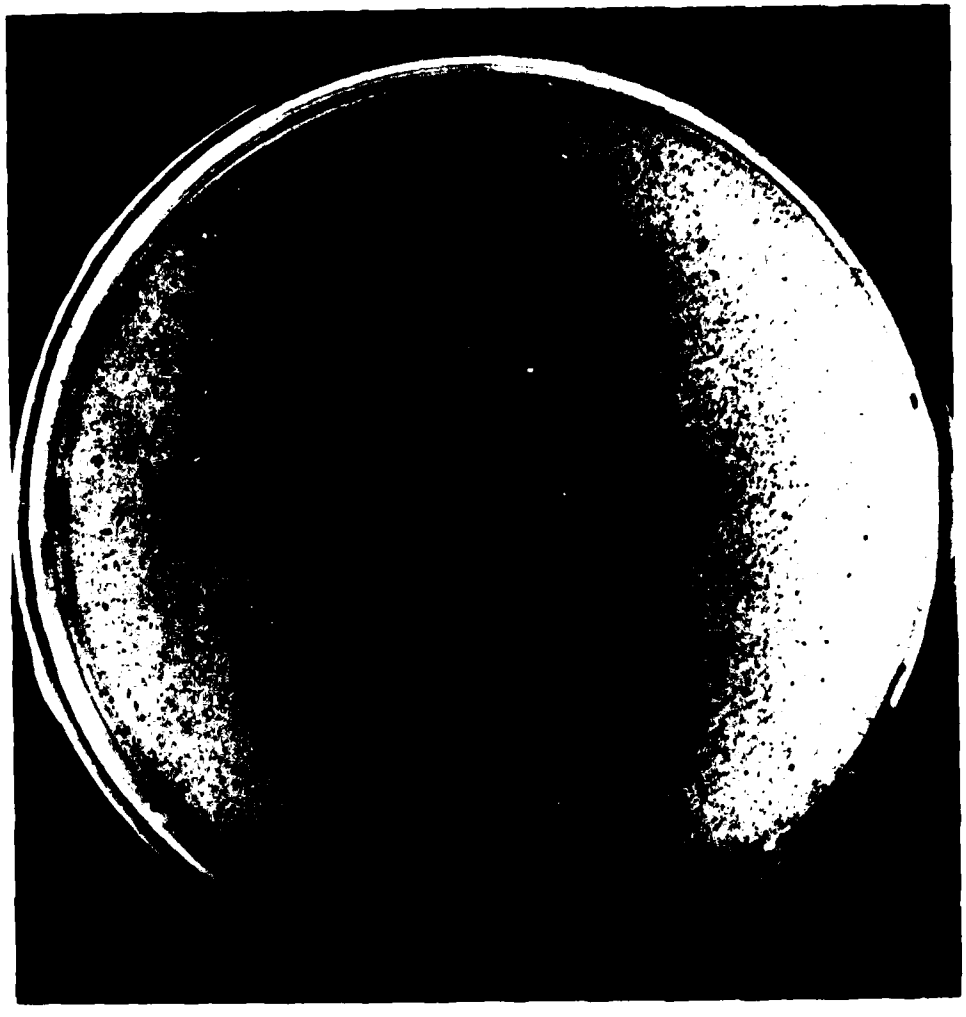

Figure 6. Plate Incorporation Test of Frozen Beef, Wild Type, S. typhimurium

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